CIRCULATORY EFFECTS OF THE VENOM OF THE INDIAN COBRA *(NAIA NAIA)* IN CATS

by

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In studying the liberation of histamine by snake venoms we found (1937a) that the venom of the Indian cobra (*Naia naia*) had this action. We had not anticipated this result. The strong local action of viperine venoms, the profound peripheral circulatory shock which is the dominant feature in poisoning by the bites of vipers and the effects of the intravenous injection of these venoms in animals—all point to the liberation of histamine. The symptoms of poisoning by the bite of the cobra and most of the recorded effects of intravenous injection of the venom in animals did not suggest this action. Only recently has a depressor action of cobra venom, as a result of peripheral vasodilatation been described in dogs, rabbits and in man (Gautrelet, Halpern and Corteggiani, 1934; Laignel-Lavastine, Würmser and Koressios, 1934).

The demonstration of the liberation of histamine from the perfused lungs of cats and guinea-pigs by cobra venom, necessitated the re-examination of its action on the circulation. In cats a fall in systemic pressure with fluid loss from circulation is a feature in poisoning both by histamine itself and by cell-injurious agents, including certain snake venoms, which liberate histamine. Such snake venoms also cause constriction of the pulmonary vessels and characteristic morbid changes in the lungs. The experiments recorded here were made to discover whether cobra venom also can cause these effects.

METHODS.

The experiments were made on cats anaesthetized with "dial" liquid (Ciba), 0·5 c.c. per kg. being injected intraperitoneally. In a few experiments the cats were anaesthetized with a chloroform-ether mixture, followed by intravenous injection of chloralose (80 mg. per kg.). Both vagi were cut, a tracheal cannula was inserted and artificial ventilation was commenced. The arterial blood pressure was recorded from the left carotid artery. The pressure in the pulmonary artery was recorded using the method described by Kellaway and LeMessurier (1936). The upper and lower lobes of the left lung were removed and a cannula was tied into the stump of the lung artery and connected with a mercury manometer. In some experiments the pressure in the corresponding lung vein was recorded simultaneously, the cannula in the vein being connected through a water manometer to a Brodie tambour. In a few experiments the pressure in the portal vein

was recorded. The spleen was removed and a cannula was tied into the splenic or pancreatic vein, the recording arrangements being the same as those for the pressure in the lung vein. Unless otherwise stated the cannulas were filled with saturated sodium sulphate.

Loss of fluid from circulation was estimated by determining the haemoglobin percentage. We used the Sahli method, the technique for collecting blood being the same as that described in the previous communication (1937b).

In order to study the effect of the venom on the vessels of the hind limb we used the method of Feldberg and Schilf (1930). The blood was made incoagulable by the injection of 1 c.c. per kg. of 8 p.c. "Chlorazol fast pink". The venom was injected into the femoral artery by a cannula tied into a side branch and the blood flowing out from the femoral vein was measured by a volume recorder and returned into the animal by a jugular vein.

In some experiments the histamine content of the lungs was determined using the method of extraction we have described elsewhere (1937a). In all experiments in which the weights of the two lungs have been compared, the small lobe situated distal to the heart (lobus intermedius) is included with the left lung. If this is done the weights of the two sides are, as we have shown (1937b) approximately equal.

EXPERIMENTAL.

Effect on Pressure in the Carotid Artery.

In cats anaesthetized with dial a profound fall in carotid blood pressure usually follows the intravenous injection of cobra venom in doses of 0·2 mg. per kg. or more. In a few instances this fall was preceded by a slight pressor response. Doses of venom smaller than 0·1 mg. per kg. had no immediate action on the blood pressure though a late gradual fall was sometimes observed, especially if the injection was repeated. There are striking individual differences in the sensitivity of cats to the depressor action of the venom. These are related to weight and age, kittens and small cats being much less sensitive than older and heavier animals.

Fig. 1 shows the effect of 0·5 mg. per kg. of cobra venom. There is a steep fall of arterial pressure, the pulsatory oscillations on the mercury manometer cease and death occurs within a few minutes. In other instances, especially if smaller doses of venom are injected, the fall in systemic pressure is not quite so steep, the blood pressure does not reach such a low level and rises three to four minutes after the injection. This rise which is often abrupt may surpass the original level. It is sometimes accompanied by quickening and strengthening of the heart beats. The pressure may remain high for many minutes, and when it falls again, does so gradually. Such an experiment is illustrated in Fig. 2. In other experiments the intermediate high level was not maintained (cf. Fig. 3). In spite of artificial ventilation the blood may not be fully arterialized.

With successive doses of venom desensitization was not complete, though a second dose after recovery of the systemic blood pressure caused only a small immediate fall. If 1 mg. per kg. or more was injected as a second dose while the blood pressure was still at a low level, a steep but evanescent rise sometimes occurred, followed by a fall terminating shortly in death (cf. Fig. 4B).

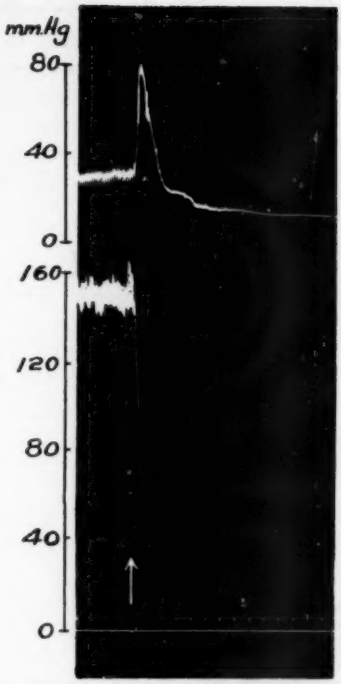

Fig. 1. Pulmonary artery (upper) and carotid (lower) blood pressure tracings from a 3 kg. cat under dial. At the arrow 0·5 mg. per kg. of cobra venom intravenously. Time in half minutes.

Effect of Chlorazol Fast Pink. In our earlier experiments, in which the pressure in the pulmonary artery and vein were recorded simultaneously, chlorazol fast pink was used for filling the cannulas or injected intravenously. These cats were less sensitive to the depressor action of the venom, doses of 1 mg. per kg. or less causing no immediate steep fall of pressure. On the other hand the pressor response to a second large dose of venom was especially striking. In the experiment of Fig. 4 the pressure fell gradually after a first dose of 1 mg. per kg. at A., and rose after a second dose of 2 mg. per kg. at B. In other experiments the first injection of 1 mg. per kg. of venom did not even produce a gradual fall of systemic pressure. In order to obtain a sharp immediate fall of systemic pressure doses of 2 mg., or more, per kg. were required.

Effect of Chloralose. Under this anaesthesia, without the administration of chlorazol fast pink, the immediate and late depressor effects of the venom were less pronounced than in cats under dial. Doses of 0·2 mg. per kg. were ineffective, but those of 0·5 mg. per kg. generally produced a steep fall of systemic pressure frequently terminating in death within a few minutes.

Analysis of the depressor action of the venom has shown that we must distinguish between those factors causing the immediate steep fall of pressure and those responsible for the later changes.

A. ANALYSIS OF THE IMMEDIATE DEPRESSOR EFFECT.

The immediate steep fall in systemic pressure is accounted for by obstruction in the pulmonary circulation without the participation of hepatic obstruction, peripheral vasodilatation, or heart failure. The depressor action of cobra venom differs, therefore, from that of the venom of the Australian copperhead, in which peripheral vasodilatation plays the dominant rôle (Kellaway and LeMessurier 1936).

Effects on Pulmonary Circulation. We have recently (1937a) shown that injection of cobra venom into the artery of the artificially perfused cat's lung causes vasoconstriction. This effect occurs also *in vivo*, resulting in a rise of pressure in the pulmonary artery which precedes or accompanies the initial fall in systemic pressure. In Fig. 1 the pressure in the pulmonary artery rose from about 30 to about 80 mm. Hg. In this experiment the pressure in the pulmonary artery was not maintained but fell to about the same level as the systemic pressure.

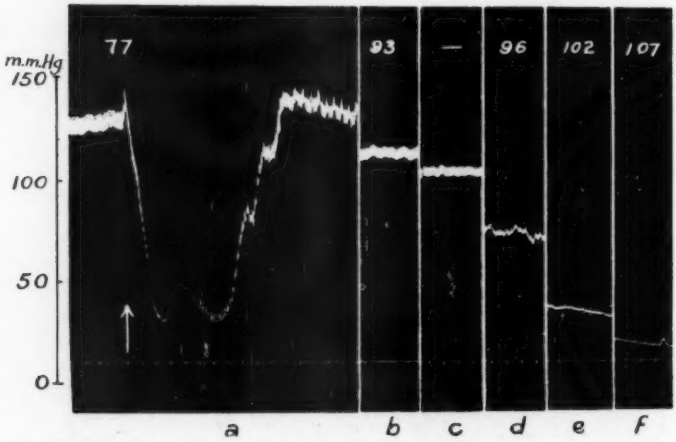

Fig. 2. Carotid blood pressure tracing from a 4·6 kg. cat. Left lung removed. At the arrow 0·2 mg. per kg. of cobra venom intravenously. b, c, d, e and f = 20, 30, 40, 60 and 85 minutes after the injection. The figures above the tracing are the corresponding haemoglobin percentages. Time in half minutes.

In other experiments (cf. Fig. 3) the pressure in the pulmonary artery remained high for several minutes and only fell gradually. At later stages it was sometimes at between 30 and 40 mm. Hg. when the systemic pressure had fallen below 20 mm. Hg. Furthermore, vigorous pulsations were to be seen on the record from the pulmonary artery, whereas the record from the carotid artery was a thin line without visible pulsations. If in such an experiment the thorax was opened before death occurred, the right heart was found to be distended and beating vigorously. The left heart was almost empty but still contracting. The left auricle was actually drawn in during diastole of the ventricle. An incision into it produced little bleeding, whereas, if the right heart was incised the thoracic cavity was flooded with venous blood. We have described a similar condition which is characteristic of extreme obstruction in the pulmonary circulation after the injection of large doses of copperhead venom (1937b).

Further evidence that the immediate rise in pulmonary pressure after venom is caused by obstruction in the pulmonary circulation and not contributed to by

failure of the left heart was afforded by experiments in which the pressure in the lung vein and artery were recorded simultanously. When the pressure in the pulmonary artery rose, that in the corresponding vein fell (Fig. 3). The changes of pressure began first in the pulmonary artery, then in the vein and last in the carotid.

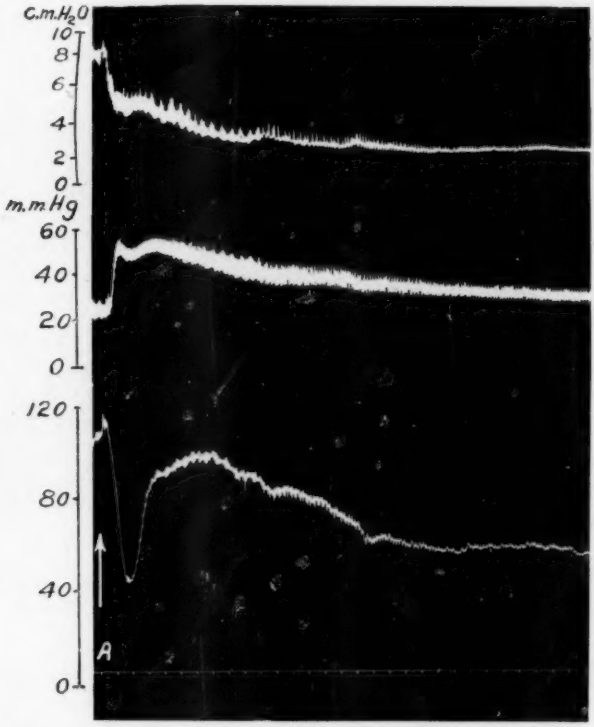

Fig. 3. Pulmonary vein (upper), pulmonary artery (middle) and carotid (lower) blood pressure tracings from a 3 kg. cat under dial. At A. 0·5 mg. per kg. of cobra venom intravenously. Time in half minutes.

In cats, after treatment with chlorazol fast pink, the injection of venom in doses up to 1 mg. per kg. caused, as we have mentioned, no abrupt fall of systemic pressure. With these doses, there was also no rise in pressure in the pulmonary artery, but doses sufficiently large to cause a precipitous fall in systemic pressure produced also a rise of pressure in the pulmonary artery. These experiments emphasize the dependence of the depressor reaction of the venom on vasoconstriction in the lungs.

In cats, which had received no chlorazol fast pink, the striking individual differences in sensitivity to the depressor action of the venom are similarly explained.

In those cats, in which after 0·2 mg. of venom per kg. no rise occurred in the pressure of the pulmonary artery, there was no fall in systemic pressure. When the fall in the latter was slight and gradual, the pressor response in the pulmonary

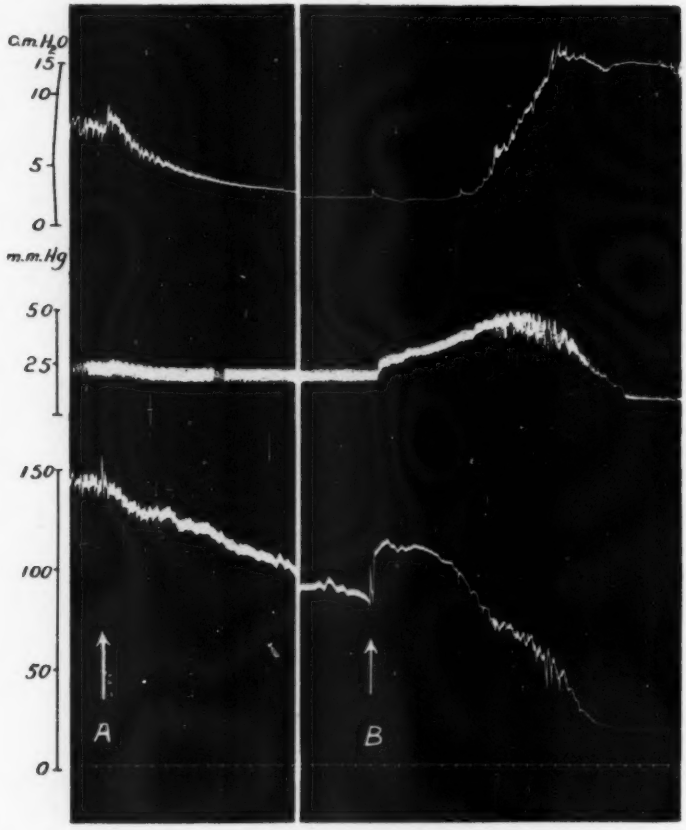

Fig 4. Pulmonary vein (upper), pulmonary artery (middle) and carotid (lower) blood pressure tracings from a 3·3 kg. cat under dial. At A. 1 mg., at B. 2 mg. per kg. of cobra venom. Between the two records 7 minutes have elapsed. Time in half minutes.

artery was small, whereas a strong pressor response in the pulmonary artery was always associated with a precipitous fall in systemic pressure.

Cats, the left lung of which had been removed, were more sensitive to the depressor action of the venom than those with intact lungs. This also points to obstruction in the pulmonary circulation as the determining factor in the depressor effect.

Effects on Portal Circulation. Histamine has at most only a feeble effect on the vessels of the cat's liver (see Feldberg and Schilf, 1930). After intravenous injections of histamine, Feldberg (1929) usually observed either no change or a fall in portal pressure; in a few instances a slight delayed rise was observed. The

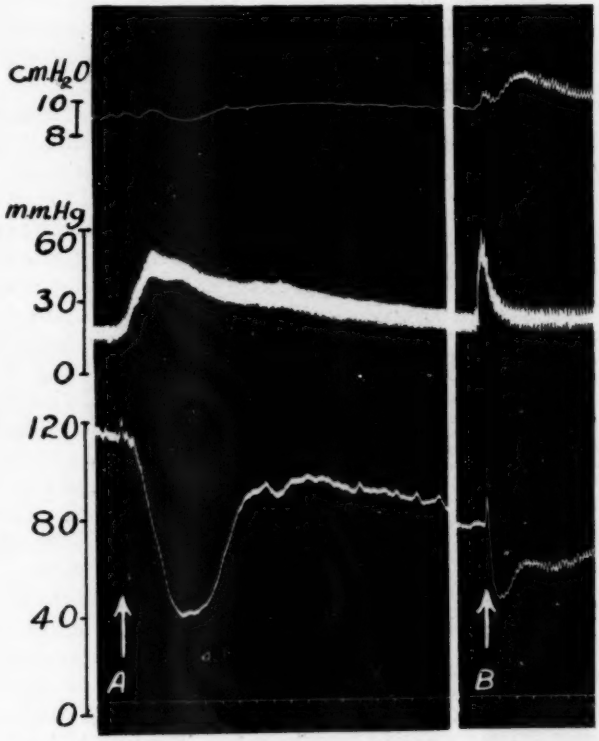

Fig. 5. Portal vein (upper), pulmonary artery (middle) and carotid (lower) blood pressure tracings from a 2·2 kg. cat under dial. At A. 0·5 per kg. of cobra venom, at B. 0·1 mg. per kg. of histamine intravenously. Time in half minutes.

results of intravenous injections of cobra venom varied in different cats, but did not indicate a constrictor effect on the hepatic vessels. The two extreme possibilities which we observed are illustrated in the experiments of Figs. 5 and 6. In Fig. 5 the portal pressure remained unchanged, in Fig. 6 it rose nearly 15 cm. of water after the intravenous injection of 0·5 mg. of venom per kg. This was probably not an effect of the venom on the liver vessels. Such large rises in portal pressure only occurred when the pressure in the pulmonary artery rose steeply and oscillatory pulsations in the systemic pressure had disappeared, indicating

strong obstruction in the pulmonary circulation. It seemed likely therefore that the rise in portal pressure resulted from back pressure in the right auricle. Evidence for this assumption was afforded by the fact that incision into the right auricle when the portal pressure was at its height, caused a sudden gush of blood from the incision, with a simultaneous steep fall of the pressure in the portal vein to below its initial level (Fig. 6B).

In the experiment of Fig. 5, 0·1 mg. of histamine per kg., injected intravenously at B., resulted in a small delayed rise of pressure in the portal vein, which is possibly also secondary to obstruction in the lung circulation.

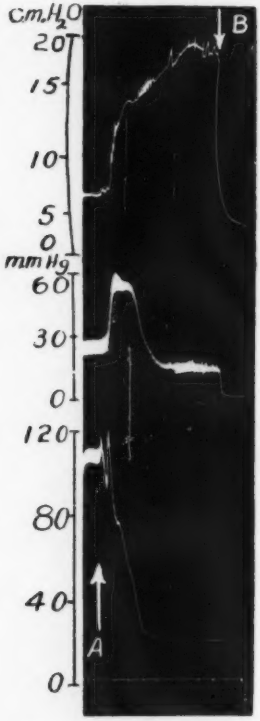

Fig. 6. Portal vein (upper), pulmonary artery (middle) and carotid (lower) blood pressure tracings from 3·7 kg. cat under dial. At A. 0·5 mg. per kg. of cobra venom intravenously. At B. incision into the right auricle. Time in half minutes.

Effects on Vessels of Peripheral Circulation. The participation of peripheral vasodilatation in the immediate depressor effect of the venom was excluded by direct determination of the effect of the venom on the peripheral vessels of the hind limb.

Under conditions of artificial perfusion "vasodilator substances" may exhibit vasoconstrictor responses. Such a "paradox" complicated the analysis of the depressor action of histamine (Dale and Richards, 1918). The method we used excluded this complicating factor; histamine invariably produced vasodilatation, whereas the venous outflow from the limb decreased after the arterial injection of cobra venom. This is illustrated in Fig. 7 in which 0·4 mg. of venom in 0·2 c.c. saline was injected. It will be seen that the vasoconstriction caused a slight rise of blood pressure. The constriction was at its height during the first minute after the injection and then diminished gradually. The venous outflow returned to normal after between five and six minutes and sometimes was slightly increased. We have not determined whether this has to be regarded as after-dilatation of vasoconstriction or as a genuine venom effect. In any case it cannot have contributed to the initial depressor reaction. The possibility of a late dilator effect will be discussed on page 170.

Vasoconstriction can be repeatedly produced by successive doses of venom and the response appears to become even more pronounced during the later stages when the systemic blood pressure is at a low level. Doses smaller than 0·1 mg. are ineffective, and for this reason the constrictor effect is not

noticeable on the blood pressure record after a first intravenous injection of venom. However, as we have seen, a second large dose may cause a pressor response especially when pulmonary vasoconstriction is prevented by chlorazol fast pink.

In one experiment we have examined the venom of the Australian copperhead by the same method. It caused increase in the venous outflow, whereas cobra venom had caused a decrease in the same animal.

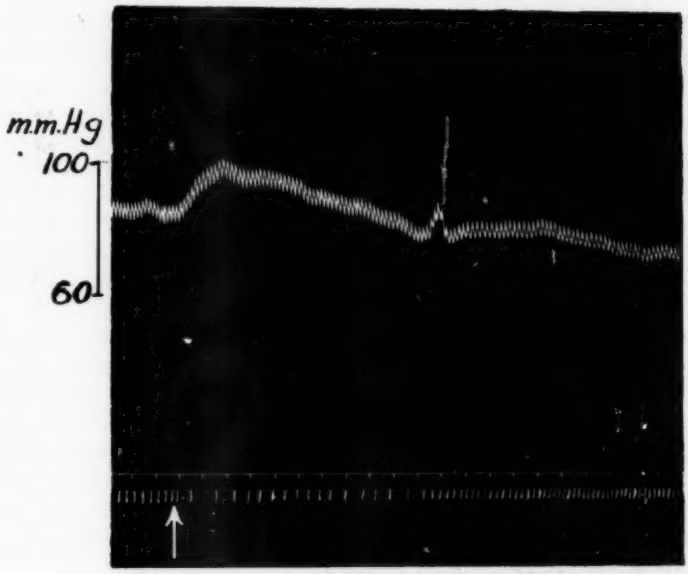

Fig. 7. Carotid blood pressure (upper tracing) from a 2·9 kg. cat under dial. Venous outflow from the hind limb (lowest tracing); each signal represents an outflow of 0·2 c.c. of blood. At the arrow injection of 0·4 mg. of cobra venom into femoral artery. Time in half minutes.

We have not studied the venous outflow from the abdominal viscera after arterial injections of venom. Evisceration did not influence the depressor reaction. Vasodilatation in the splanchnic area could therefore be excluded as contributing to the immediate depressor action of the venom.

B. ANALYSIS OF THE LATE CHANGES IN SYSTEMIC BLOOD PRESSURE.

Several factors contribute to the late gradual fall in systemic pressure. Weakening of the heart, haemorrhagic lung oedema and peripheral vasodilatation with fluid loss from circulation appear to be mainly responsible.

Heart Changes. We have mentioned that the initial steep fall in systemic

pressure is not accounted for by changes in the heart action, as evidenced by the accompanying fall of pressure in the lung vein. In some instances, a few minutes after the injection of large doses of venom, when the systemic pressure had already fallen to a low level and the pressure in the pulmonary artery had risen over 40 mg. Hg., we have observed a sudden falling-off of this pressure. This may have been due to failure of the right heart to overcome obstruction in the pulmonary circulation. In other instances, in which less venom was injected, the fall in systemic pressure was interrupted a few minutes after the injection by a rapid rise of pressure, which was sometimes accompanied by tremendously increased force and rate of the heart action which was possibly the result of adrenaline output.

The late gradual fall in systemic pressure is not always attended by obvious signs of heart failure, but sometimes the heart becomes slow and feeble, dropped beats or other cardiac irregularities occur and death then usually follows within half an hour. Acute heart failure generally results a few minutes after the injection of a second large dose of venom (2 mg. or more per kg.). This is shown in Fig. 4B. by the rise in pressure in the lung vein which accompanies the onset of cardiac irregularity and the final fall of pressure in the pulmonary and carotid arteries.

We have not analysed the cardiac changes. They are probably direct effects of the venom, which is known to be powerfully toxic to the isolated heart (Gunn and Heathcote, 1921; Manwaring and Williams, 1923; Kellaway and Williams, 1932).

Lung Changes. The changes in the lungs following the injection of cobra venom are of similar kind but more intense than those caused by the venom of the Australian copperhead (Feldberg and Kellaway, 1937b). With the steep fall in systemic pressure the respiratory movements of the ventilated lungs decrease or cease in expiration. If the cat dies within a few minutes after the injection the lung is collapsed and has a tough, fleshy consistency. Microscopically there is atelectasis and congestion. If the cat survives for a longer period the respiratory movements recommence or increase, and intense haemorrhagic oedema develops. When the thorax is opened an hour or more after the injection, the lungs are distended and relax only slightly during the intervals between each inflation. They are bright red in colour and exhibit collapsed patches and areas of haemorrhage. The peribronchial lymph vessels may be extremely swollen. The lung weight is found to have increased twice to four times and fluid drains from the cut surface. In Table 2 the amounts of oedema fluid accumulated in the right lung are given. The first few c.c. of fluid draining from the lung contain blood, but later samples are clearer, though tinged by haemolysis. On page 170 the haemoglobin concentration is given of two successive samples of drainage fluid from the lung of the experiment of Fig. 2. These changes account for the failure of the lungs to arterialize the blood. The pulmonary changes are not so extensive in kittens and young cats as in older and large cats, which have a higher histamine content in their

lungs. A similar observation has been made for the lung changes induced by the venom of the Australian copperhead.

Fluid Loss from Circulation. After the injection of venom, fluid escapes from circulation causing an increase in the haemoglobin concentration of the blood. This is illustrated in Table 1. In the first three experiments, from cats under dial anaesthesia, the increase in the haemoglobin concentration after venom amounted to between 35 and 53 p.c. The haemoglobin concentration rose gradually in successive samples, indicating a progressive fluid loss from circulation. The record of the systemic blood pressure in the first experiment of this table is shown in Fig. 2. Fluid loss had already commenced, when the pressure was still at a high level.

TABLE 1.

Haemoglobin Concentration of Blood After 0·2 mg. per kg. of Cobra Venom.

No. of Experiment.	Body weight in kg.	Haemoglobin. Before venom.	Minutes after venom 20–25	30–35	40–45	60–65	80–90	Maximal increase in Hb. (in p.c.).
1 (Exp. of Fig 2)	4·6	77	93	—	96	102	107	39
2	4·2	69	78	78	93	—	—	35
3	2·7	45	—	60	65	69	—	53
4	3·6	92	113	116	125	125	—	36
5	4·1	64	69	72	74	75	—	17
6	4·1	77	87	87	86	83	—	13

From the maximal increase in the haemoglobin concentration and the body weight the amount of fluid lost from circulation was estimated. Dale and Laidlaw (1919) have shown that cats have about 50 c.c. of blood per kg., a third of which constitutes the corpuscle volume. This ratio is constant in spite of great individual variations in the haemoglobin value. An increase in the haemoglobin concentration of 39 p.c., as in experiment 1 of Table 1, corresponds therefore to an increase in the corpuscle volume from 33·3 to 46·3 p.c., which in a 4·6 kg. cat with a blood volume of 230 c.c. would be brought about by a fluid loss of 65 c.c. The fluid loss calculated in the same manner for the other experiments of Table 1 is given in the second column of Table 2.

TABLE 2.

Fluid Loss After 0·2 mg. per kg. of Cobra Venom.

No. of Experiment.	Calculated fluid loss in c.c.	Fluid loss in right lung in c.c.	Fluid loss in right lung as percentage of total loss.
1	65	29	45
2	55	14	25
3	47	10	21
4	48	18	38
5	28	10	35
6	25	2	8

Lung oedema accounts for part of this fluid loss. In the experiments of Tables 1 and 2 the left lung had been removed before the venom was injected. From the

difference in weight of the lungs (see Methods) the fluid accumulation in the right lung was estimated, allowance being made for the fluid which had drained from it. For instance, in experiment 1 the control left lung weighed 13·9 gm. and the right 27 gm. after 18 c.c. of fluid had drained off in fifteen minutes. This fluid had been collected in two successive samples. The first sample of 11 c.c., draining during three minutes, had a haemoglobin percentage of 18, the second sample of 7 c.c. one of 8 p.c. only. The haemoglobin content of the whole 18 c.c. corresponded therefore to about that of 2 c.c. of blood with a haemoglobin percentage of 107, as found in the last sample of blood removed from the femoral artery. Thus 16 c.c. of the 18 c.c. of fluid could be regarded as oedema fluid, to which about 13 c.c. (the difference of the lung weights) must be added, bringing the fluid accumulation in the right lung to 29 c.c. or 45 p.c. of the total fluid loss. If the left lung had been left *in situ* another 29 c.c. might possibly have been lost from circulation. In this case the lungs would have accounted for 62 p.c. of the total fluid loss. In the same way the fluid accumulated in the right lung has been estimated in the other experiments (columns 3 and 4 of Table 2) and accounts for only part of the fluid loss.

Other possible causes of the increased haemoglobin concentration—contraction of the spleen, accumulation of fluid in the liver and increased permeability of the capillaries—must therefore be considered. Experiments 4 and 5 of Tables 1 and 2 were carried out, under dial anaesthesia, on cats, in which the stomach, small and large intestine and spleen had been removed and the hepatic artery tied. In these animals also lung oedema accounted only in part for the calculated fluid loss from circulation. There must therefore be increased permeability of the capillaries and it appears unlikely that this is achieved without concomitant vasodilatation.

We have made a few experiments in cats under chloralose, in which condition cobra venom has a less depressant effect. We found that the fluid loss from circulation and the lung oedema, were less than under dial anaesthesia, and that the fluid loss was not so long continued. This is illustrated by experiment 6 (Tables 1 and 2) from a cat under chloralose. There is actually a decrease in the haemoglobin concentration in the last two samples. This is probably accounted for by the removal of the blood samples since we have observed a similar small decrease in taking successive samples of blood from normal cats.

DISCUSSION.

The experiments here recorded show that in the cat the intravenous injection of cobra venom may cause a profound fall of systemic pressure with loss of fluid from circulation. The immediate depressor effect is not the result of peripheral vasodilatation but is accounted for by pulmonary vasoconstriction. After the injection of moderate doses of the venom of the Australian copperhead the immediate fall of blood pressure is brought about by peripheral vasodilatation, and it is only with large doses that obstruction of the pulmonary vessels contributes to this effect and may determine the fatal issue. Though the pulmonary vasoconstriction caused

by the venom of the Australian copperhead may be attributed to the liberation of histamine in the lung, this mechanism cannot wholly account for the obstruction in the pulmonary circulation after cobra venom, because we have observed (1937a) vasoconstriction after this venom in the perfused guinea-pig's lungs, the vessels of which do not respond to histamine. To what extent liberation of histamine intensifies pulmonary constriction after cobra venom in the cat, remains uncertain. Cobra venom has a strong constrictor action upon other vessels, and this may be of similar nature to that causing obstruction in the pulmonary circulation.

The lung vessels of kittens and young cats, which have a low histamine content in their lungs, are relatively insensitive to the vasoconstrictor action, as also to the directly cell-injurious effects of cobra venom. They are also relatively insensitive, as we have shown previously (1937b), to histamine. There appears to be a general association between high histamine content of the lungs, high sensitivity of the lungs to cell injury and of their vessels to histamine, to cobra venom and possibly to other substances which cause pulmonary vasoconstriction.

If the pulmonary vasoconstriction caused by cobra venom is overcome and the systemic blood pressure recovers, a secondary gradual fall of systemic pressure may occur associated with blood corpuscle concentration—a cardinal feature of histamine shock. Oedema of the lung accounts only for part of this effect and the remaining part cannot be explained either by contraction of the spleen or by accumulation of fluid in the liver. Fluid loss from the circulation brought about by increased permeability of the capillaries must therefore take place, either directly by peripheral cell-injurious action of the venom or by the resulting local liberation of histamine or by both. There is still another possible explanation. Histamine liberated in the lungs may escape into the circulation in amounts sufficient to cause these late effects. If this were so the cat's lung would represent a typical "shock organ" for cobra venom and the difference between the circulatory effects of this venom and those of the Australian copperhead would lie in the fact that the cell-injurious action of the latter venom affects many organs. In this event the action of cobra venom would resemble that of staphylococcal toxin, the depressor action of which in cats is accounted for wholly by obstruction in the pulmonary circulation (Kellaway, Burnet and Williams, 1930). The lesions caused in the lungs by staphylococcal toxin are localized round the bronchi and great vessels, whereas those observed after the injection of cobra venom are widespread throughout the lungs.

The sensitivity of the lung vessels to cobra venom is widely different in different species. We found recently (1937c) that in the dog, the lung vessels were insensitive to this venom. Though the changes observed in the lungs after death from snake bite make it likely that the lung vessels in man are sensitive to cobra venom, we have no direct evidence on this point. In any case the effects on the systemic blood pressure caused by pulmonary vasoconstriction could hardly be expected to occur after snake bite in man unless the fangs were to enter a vein, because rather large intravenous doses are necessary to produce obstruction in the

pulmonary circulation. The absence of circulatory shock in human beings bitten by the cobra is probably also dependent upon the predominantly curari-like action of this venom. Kellaway and Holden (1932) have shown that in frog muscle preparations the curari-like action of cobra venom is more than 20 times stronger than that of the venom of the copperhead, and we have found (1937a) that the venom of the Australian copperhead is about 4 to 5 times as active as cobra venom in liberating histamine from perfused lungs. After snake bites by the cobra, curarization of the diaphragm and failure of respiration probably take place before the circulatory shock observed in artificially ventilated animals has had time to develop. Our experiments show that cobra venom does not differ essentially from venoms like that of the Australian copperhead which produces conditions of circulatory shock.

SUMMARY.

1. In cats, under dial anaesthesia, intravenous injection of cobra venom (0·25 — 0·5 mg. per kg.) causes an immediate steep fall of systemic pressure. Death may occur within a few minutes. If recovery of the blood pressure takes place there is a secondary gradual fall, resulting eventually in death. In cats, under chloralose, larger doses of venom are necessary to produce these effects.

2. The immediate steep fall of systemic pressure is accounted for by obstruction in the pulmonary circulation; peripheral vasodilatation, constriction of the liver vessels and heart failure do not contribute to it. The steep fall in systemic pressure is accompanied or preceded by a rise of pressure in the pulmonary artery and a fall of pressure in the pulmonary vein. Venom injected into the femoral artery causes immediate vasoconstriction in the hind limb.

3. The late gradual fall of systemic pressure is accounted for by fluid loss from circulation, probably associated with peripheral vasodilatation, haemorrhagic oedema of the lungs and, in some cases, failure of the heart action.

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STUDIES IN THE GROWTH AND RESPIRATION OF TOMATO FRUITS AND THEIR RELATIONSHIP TO CARBOHYDRATE CONTENT

by

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INTRODUCTION.

The study of the respiratory curves of fruits has come into prominence during the last few years and many investigators have demonstrated the presence of a climacteric rise in the respiratory curve during the senescent phase.

The growth and respiratory curves of tomato fruits have previously been worked out by Gustafson (1926 and 1929), who has shown that the climacteric rise occurs in tomatoes and is coincident with the appearance of colour in the fruit.

Intensive investigation on apple respiration has been carried out by Kidd and West (1930), and also by Blackman (1928). The former found that the maximum respiratory intensity is approximately 1·5 times the initial value. Blackman similarly demonstrated the climacteric rise in apples. Furthermore, he divided apples into three main physiological groups according to their rate of ripening. Olney (1926) demonstrated the presence of a climacteric rise in bananas.

The investigation on tomatoes under discussion in this paper revealed great fluctuations in the respiratory intensity, carbohydrate content and growth rate. Gustafson's work exhibits similar variation, so that an explanation for these fluctuations was sought. By carefully noting the position of the fruit on the plant and the number of fruits per truss, the important conclusion has been drawn that difference in respiratory intensity, carbohydrate content and growth rate can be attributed to the position of the fruit on the plant relative to the food supply and to the other fruits. Since tomatoes bear their fruits in trusses in which the flower nearest the main axis is usually fertilized first and that furthest away from the main axis is fertilized last, they provide excellent material for study.

MATERIAL.

The fruits used throughout were those of the Kondine tomato, grown at Ryde in a glass-house during the winter and early spring of 1936. A complete series was run with fruits which received no artificial heat, the mean minimum temperature being approximately 4°C. and the mean maximum temperature being about 24°C.

A comparison was made with fruits supplied with artificial heat. These were cultivated in adjoining glass-houses during the same months of the same year, but the houses were heated by means of hot water pipes. The mean minimum temperature was approximately 15°C. and the mean maximum 27°C. In both cases the plants were grown directly in the soil (not in pots) and received ample water.

Since great variations were found to occur in the growth rate and maximum size of the plant in different parts of the glass-house, all fruits chosen for analysis and respiratory determinations were taken from plants near the centre of the house, where growth was most rapid.

ANALYTICAL METHODS.

Respiratory Intensity.

The respiratory intensity was measured at 25°C., and is expressed in every case as mg. CO_2 per 100 gm. of fruit per hour.

The fruits were enclosed in specimen jars which were tightly sealed with rubber bungs. The jars were submerged in a water-bath whose temperature was kept constant at 25°C. ($\pm$ 0·2) by means of a gas regulator. The amount of carbon dioxide evolved was estimated by absorption in standard caustic soda (approximately one-tenth normal) in Pettenkofer tubes. The carbonate was precipitated with barium chloride and the excess soda was titrated with standard hydrochloric acid. Individual fruits were used except in the case of the young stages, where, owing to the small mass, the number of fruits and the time period were increased. With larger fruits, a time period of three to five hours was necessary to give a sufficient amount of carbon dioxide for an accurate reading.

During ripening, the course of the respiratory curve was carried out on the same fruit. Daily readings were done, at regular intervals of twenty-four hours, the time period for each reading being kept constant from day to day. During the remainder of the time, a stream of carbon dioxide-free air was drawn over the fruit to prevent the accumulation of carbon dioxide in the respiratory jar. Careful blanks were run during the estimations, and in every case the amount of carbon dioxide absorbed from the atmosphere was extremely small.

Carbohydrate Estimations.

1. *Extraction.* Soluble carbohydrates were extracted with boiling 70 p.c. alcohol. The alcohol was removed and all determinations were done in aqueous solution. The fruits were cut finely, and the sugars were extracted by boiling with four small quantities of alcohol in a boiling water-bath under a reflux condenser.

2. *Total Reducing Sugars.* Total reducing sugars were determined by the Lane and Eynon (1923) copper-reducing method using methylene blue as an indicator. This method was chosen since large numbers of reducing sugar determinations were done and a quick method was therefore essential.

3. *Clearing of Solutions.* Neutral lead acetate, basic lead acetate and charcoal were tested as clearing agents. Heavy losses of sugar occurred when the first of these was used. The latter two gave almost identical results. Charcoal was preferred on account of its being more rapid. The loss of sugar during charcoal clearing is very small: 100 c.c. of an aqueous solution containing 0·441 gm. of glucose were boiled with two grams of charcoal (a commercial preparation "Norit") for two hours. After this treatment the solution contained 0·435 gm. of glucose.

The method employed to clear the tomato extract was to boil with about 0·5 gm. of charcoal for half a minute and filter by suction. In most cases a second treatment with charcoal was not necessary to produce a clear solution.

4. *Starch.* Starch was determined in the alcohol-insoluble residue by hydrolysis with taka-diastase according to the method outlined by Widdowson (1931). The glucose obtained was estimated by Lane and Eynon's method.

In every case the percentage carbohydrate is calculated on the fresh weight.

Growth.

The growth curves of the developing fruits were obtained by detaching and weighing the fruits at weekly intervals and plotting fresh weight against the time. Dry weight was measured by drying the fruits at 105°C. in an electric oven until they had reached constant weight. This usually took about one week. The fruits were tagged with string or coloured wool when the corolla commenced to wither. This was regarded as the beginning of the fruit stage. Malformed fruits and those on fungus-infected plants were rejected.

RESULTS.

A. *Young Stages.*

1. *Growth.* In a single truss of flowers, that nearest the main axis is fertilized first and the fruit derived from this flower may be a centimetre in diameter before the last flower is fertilized. These "first" fruits have been considered as a separate series, since their maximum size, respiratory intensity and sugar content are the greatest. The remaining fruits on the truss which commence to grow normally fall roughly into two groups. For this reason it has been possible to split the fruits into three groups depending on the position of the fruit on the truss. These three groups, though ill-defined, are sufficiently marked to assist in elucidating the main conclusions, and are referred to throughout as "first", "intermediate" and "last" fruits. The second, third and fourth fruits were regarded as "intermediate" and the fifth and sixth as "last" fruits. "First" fruits attain the greatest size, "intermediate" fruits being smaller, and "last" fruits being smallest (Table 1). This division has been made purely for convenience. Actually, every fruit is physiologically and chemically distinct from other fruits, but it would not be possible to consider a single fruit throughout.

In the Kondine tomato, the number of fruits per truss is large, in many cases well over twenty flowers being counted. However, it is rare to find all the fruits a normal size, the number of mature fruits usually being five or six. In addition to these, there is always, at the end of the truss, a varying number of tiny fruits which in ten weeks may be no larger than a centimetre in diameter.

The shape of the curves obtained is of the typical growth nature—a gradual rise for the first few weeks followed by a steep slope and culminating in a flattening indicating maturity. The ultimate size attained varies with each "type", thus altering the gradient of the curve. The maximum size varies greatly; "intermediate" fruits are quite often as large as "first" fruits of the same truss, while "intermediate" fruits of one truss may be as large as "first" fruits from another. The greatest variation in size is found amongst "first" fruits and is most conspicuous in fruits grown without internal heat (Table 1).

The mass of the "first" fruits which ripen in the winter is greater than that of the spring-ripening fruits. This is probably due to the fact that during the winter the number of fruits per truss is small; whereas in the spring more fruits per truss develop so that no single fruit reaches a great size, but the total mass of fruit on the spring-grown truss is greater than that of the winter-grown.

TABLE 1.

Showing the Difference in Size of Fruits from Different Parts of the Truss. All Figures are for Fresh Weight.

"First" fruits. First truss.	"Intermediate" fruits. First truss.	"Last" fruits. First truss.
104 gm.	88 gm.	19 gm.
103 „	75 „	18 „
87 „	67 „	18 „
78 „	66 „	17 „
69 „	58 „	17 „
67 „	57 „	14 „
66 „	52 „	14 „
55 „	47 „	12 „
33 „	26 „	10 „

The fresh weight growth curves of the three types are shown in Fig. 1 (Curves A, B and C); Curve D represents a series of "first" fruits from the second truss. This curve shows a similar shape to the others but the maximum size of the fruit is smaller than that of the "first" fruit of the lower truss. Each point plotted is the mean of many determinations (approximately eight). The deviation from the mean is greatest in mature fruits and least in the very young fruits. The masses of individual fruits are not indicated since the large number of points would lead to confusion.

The growth curves have been continued up to the week prior to the appearance of colour in the fruit. It can be seen from the graphs that "first" fruits mature more rapidly than those remote from the main axis.

The dry-weight curve (Fig. 2, Curve A) shows a similar shape to the fresh weight curves; but the percentage of dry weight of the fruit decreases as the fruit matures. This is shown by the following experimental figures:

The ovaries of "first" fruits were found to contain 18 p.c. dry matter. The percentage dry weight falls immediately after fertilization; fruits of one week old contained 15 p.c. dry matter. The fall was rapid during the period of rapid growth and flattened out towards the ripening period, the readings being 5·5 p.c. for the fully-grown fruits. During ripening, the percentage dry weight falls slightly; full-red fruits contained 4·5 to 5·0 p.c. The percentage dry weight curve is shown in Fig. 2, Curve B. Each of these points represents the mean of two readings which are not indicated separately since their deviation from the mean is low (not exceeding 2·0 p.c.).

In a truss of fruits grown with artificial heat, the differences between "first", "intermediate" and "last" fruits do not become very conspicuous, although there is a slight diminution in size on passing away from the main axis. These fruits were fairly uniform in size, the mean mass being about 47 gm., while the number

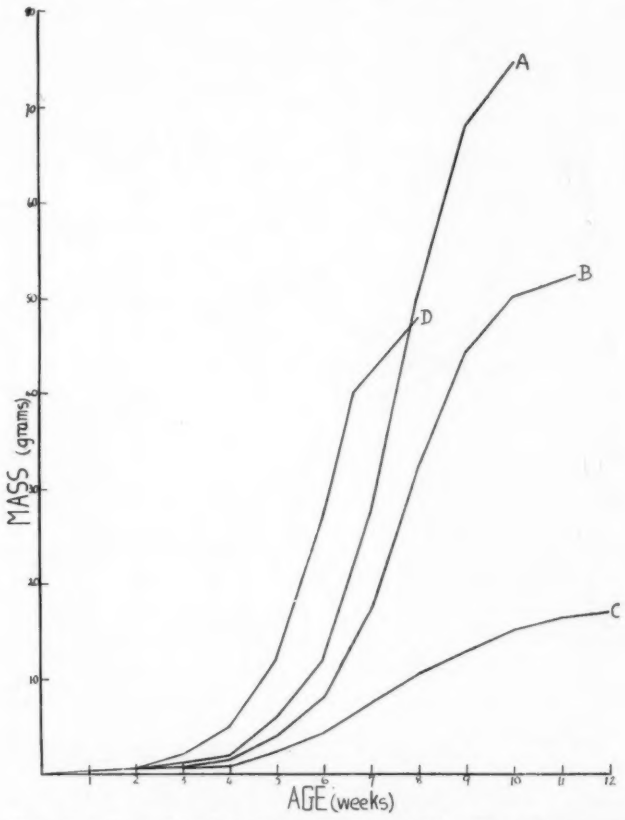

Fig. 1. Growth curves of tomato fruits (fresh weight). Curve A represents "first" fruits, curve B "intermediate" fruits, curve C "last" fruits, all three from the first truss and curve D "first" fruits from the second truss.

of fruits per truss was large (about eight to ten), so that the total mass of the fruit from the individual truss frequently exceeded the total mass of fruit from the individual truss of a plant grown without artificial heat.

This may be attributed to the fact that inside heated glasshouses the air temperature is high enough to permit the manufacture of a sufficient amount of food

material for the maintenance of a large number of fruits; whereas, in unheated houses, the occurrence of a limited number of normal and a large number of undeveloped fruits suggests that the food supply is insufficient to maintain all the

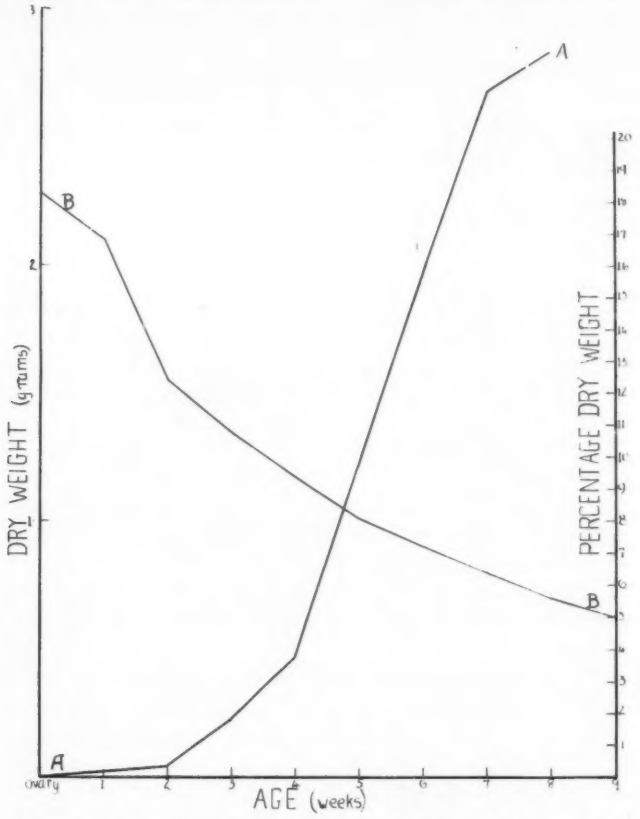

Fig. 2. Curve A shows the dry-weight curve of tomato fruits and curve B the percentage dry weight of the same fruits.

fruits on the truss so that a monopolization of the food material by the fruits nearest the food supply occurs, resulting in the growth and maturation of those fruits nearest the main axis.

3. *Respiration.* Respiration in "first" fruits alone was considered during the young stages.

Owing the minute size of the very young fruits, the respiratory activity could not be accurately estimated since relatively large cuts resulted when the fruits

were removed from the plant. At two weeks, the fruits were sufficiently large to handle. The respiratory activity of such fruits was approximately 55 mg. of carbon dioxide per 100 gm. of fruit per hour. This value fell rapidly until the fruits were about four weeks old, and from this age, the value fell more slowly, there being only a difference of approximately 4·5 mg. of carbon dioxide between the five and nine week old fruits. The value of the one week old fruits could be approximately calculated by producing the curve backwards, and would lie between 80 and 90.

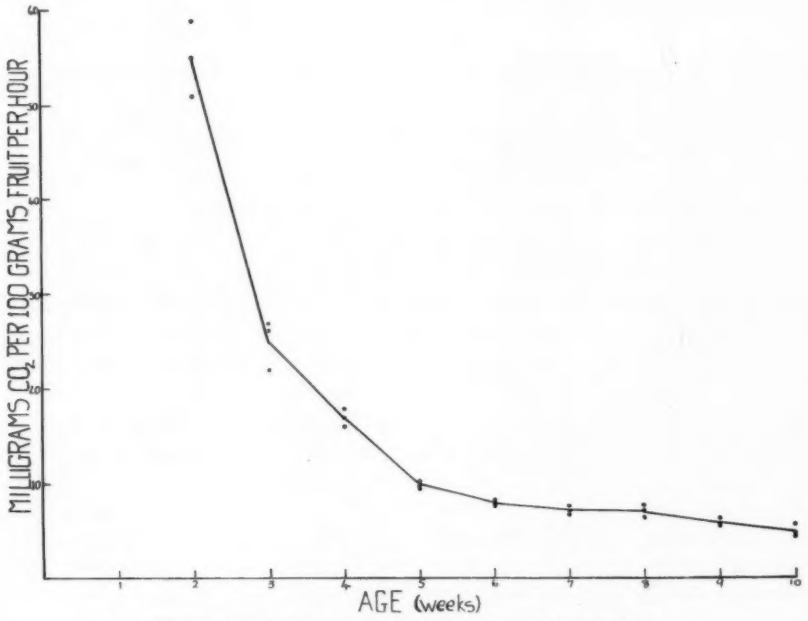

Fig. 3. Respiratory curve of developing "first" fruits.

The respiratory curve (Fig. 3) has been followed to the tenth week only, just before the appearance of colour in the fruit. It is at this stage that the climacteric rise occurs, which will be fully discussed below when the ripening process is considered.

4. *Carbohydrate Content.* The carbohydrates investigated were the reducing sugars (glucose and fructose), and starch. No sucrose or any other easily hydrolysable sugar was present. Hemicelluloses occurred, their percentage being highest in the young fruits and decreasing with age, but a complete series of readings was not carried out (see below).

After fertilization, the percentage of reducing sugars is very low. This is probably connected with the high respiratory intensity of the young fruit. At the age of one week, the fruit contained about 0·1 p.c. sugar. (This reading is only

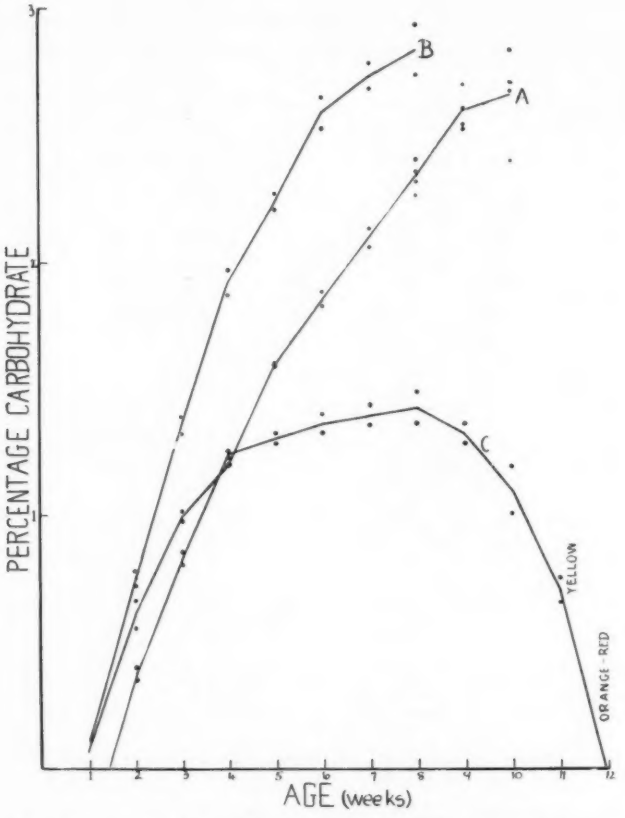

Fig. 4. Percentage reducing sugars and starch in tomato fruits. Curve A represents "first" fruits on the first truss and curve B "first" fruits on the second truss. Curve C represents the percentage starch in "first" fruits from the young to the red stage.

approximate since the fruits were very small and only a limited number was available for analysis.) As the fruit increases in size, the percentage of reducing sugars increases steadily. This increase is accompanied by the appearance of starch, which was first detected when the fruits were ten to fourteen days old.

According to Sando (1920), the percentage of starch decreases with age,

being at a maximum in the young fruits. The results given in this paper show that no starch is present in the ovaries or young fruits up to about ten days. In explanation of this discrepancy in Sando's results, it should be pointed out that he used acid for his starch hydrolysis and thus obtained readings which include both starch and hemicellulose. In this present investigation hemicelluloses were estimated separately during the young stages of the fruit and the results showed that the percentage of hemicellulose decreases with age.

The curves showing the percentage of reducing sugars and starch are given in Fig. 4.

B. *The Ripening Process.*

1. *Respiration.* With the first appearance of colour in the fruit, great changes occur both in the respiratory intensity and the chemical composition. When the fruit has reached its maximum size, its respiratory rate varies between 3 and 5 mg. CO_2 per 100 gm. of fruit per hour, depending on the "type" of fruit.

When the respiratory intensity of any such green fruit was measured from day to day, it was found that the curve, whose shape is more or less the same for all fruits, fell slightly (by approximately 0·5 mg. of CO_2). This low value was only transitory, persisting for two or three days. It was followed by a rapid rise which reached its maximum at the orange-red stage. The increase was approximately 50 p.c., but in some cases, twice the original low value was recorded. The general shape of such a climacteric rise is shown in Figs. 5, 6 and 7.

The initial and maximum values attained during the climacteric rise are by no means constant, but unexpected and wide variations were obtained. These variations are linked up with the size of the fruit and its carbohydrate content. The former of these, as was shown in the first part of the paper, is definitely associated with the position of the fruit on the truss. Thus the first fruit on the plant to mature is not only the largest, but its respiratory intensity (as well as its percentage reducing sugars) is the highest. With smaller fruits ("intermediate" and "last" fruits), is associated a lower respiratory intensity and the climacteric rise runs approximately parallel to that of the "first" fruits but at a lower level. "Last" fruits have the lowest respiratory intensity. Little difference occurs in the rate of ripening, but, as a general rule, the first-formed fruits ripen a little more quickly than the "intermediate", and these ripen a little more quickly than the "last" fruits. Such differences might become more apparent if the fruits were ripened at lower temperatures. Unfortunately it was impossible to carry out cool storage experiments.

Among the "first" fruits differences occurred in the maximum respiratory intensity and likewise in the "intermediate" and "last" fruits. These variations (shown in Table 2) can be attributed to individual differences which naturally occur.

TABLE 2.

Showing the Relationship between Position of the Fruit on the Plant and Respiratory Intensity.

Position on the truss.	Maximum respiratory intensity. Mg. CO_2 per 100 gm. of fruit per hour.
"First" fruit	7·7
"First" fruit	8·5
"Lone"* fruit	8·7
"Intermediate" fruit	7·5
"Intermediate" fruit	7·5
"Intermediate" fruit	7·1
"Intermediate" fruit	7·3
"Last" fruit	6·0
"Last' fruit	4·8
"Last" fruit	5·2

* A fruit which developed alone on a truss.

In Fig. 5, four climacteric curves are shown. Curves A and B are those of "first" fruits, curve C, that of an "intermediate" fruit, and curve D, that of a "last" fruit. From these it is quite obvious that the position of the fruit on the plant affects the respiratory intensity.

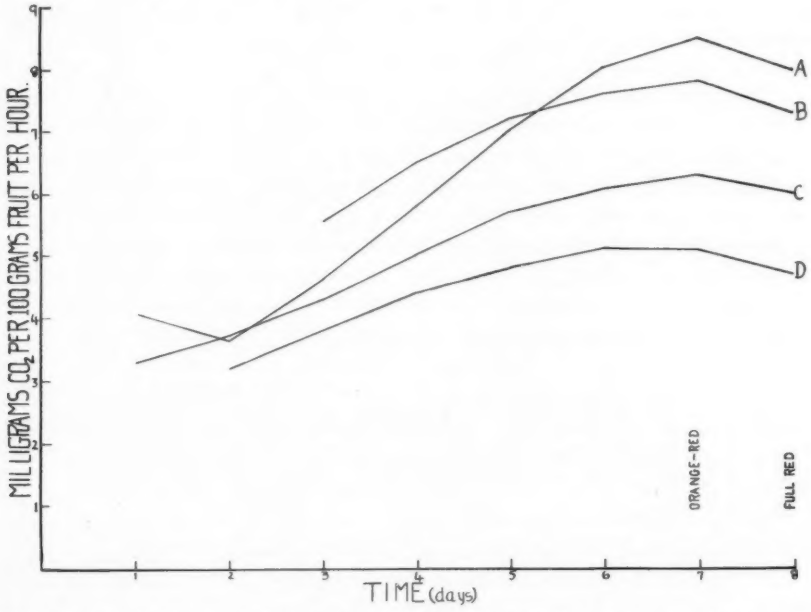

Fig. 5. Curves showing four climacteric rises of individual fruits. For explanation see text.

The climacteric curves outlined above were obtained from individual fruits, i.e. under storage conditions, but this does not necessarily prove that the climacteric rise occurred when the fruit ripened naturally on the plant. An attempt was therefore made to demonstrate the presence of the climacteric rise under natural conditions and the procedure outlined below was followed.

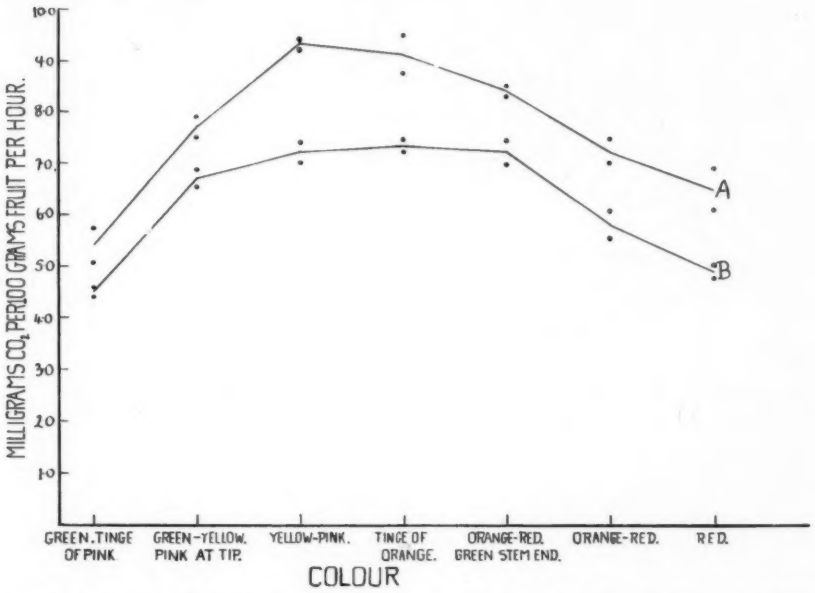

Fig. 6. Curves showing climacteric rises for freshly picked fruits. Curve A "first" fruits, curve B "intermediate" fruits.

Fruits, at distinct colour stages, were picked from the plant, removed to the laboratory and their respiratory intensity was measured. A complete series including only "first" fruits at different colour stages was run. These points were plotted on a graph (Fig. 6) and joined to give a climacteric curve. A second series of "intermediate" fruits was done, resulting in a similar curve, but at a lower level.

From these curves it may be concluded firstly, that the climacteric rise occurs under natural conditions, and secondly, that the "first" fruits have a higher respiratory intensity at all colour stages than "intermediate" fruits, which is in accordance with the data given above for stored fruits.

The colours at the various stages of ripening, exhibited by fruits which are allowed to mature on the plant, differ slightly from those of stored fruits. In the former the appearance of colour is not even, but the green colour persists at the stem end up to the orange-red stage and disappears with the development of a full red colour. In stored fruits the colour changes are: green-yellow, yellow, orange, red, with no persistence of green after the yellow stage.

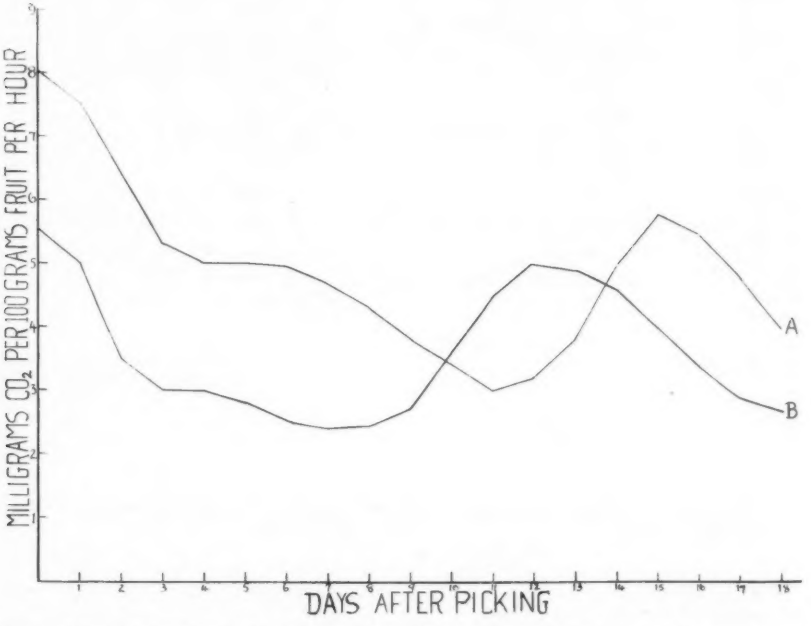

Fig. 7. Respiratory curves of prematurely picked fruits. Curve A was obtained from a "first" fruit, and curve B from an "intermediate".

The maximum value obtained during the climacteric rise was found in approximately the same position as in stored fruits but the respiratory intensity throughout was higher than that of the stored fruits, which may be attributed to the fact that the turgidity and sugar content of freshly-picked fruits were higher than for stored fruits at the same colour stage.

2. *The Effect of Premature Picking on the Respiratory Curve.* Numerous fruits were picked when about seven to eight weeks old and their respiratory activity was measured daily until they had developed a red colour. The climacteric rise was found in approximately the same position as for the late picked fruits. The initial respiratory rate was at first high (in the vicinity of 8 mg. CO_2 per

100 gm. of fruit per hour), but this rapid evolution of carbon dioxide very quickly fell (after a period of two or three days) and the respiratory activity remained fairly uniform until a few days before the appearance of colour. The curve then fell slightly, corresponding to the dip found in late picked fruits. Following the decrease, the climacteric rise was initiated and reached its peak at the yellow-orange stage (thus differing from the late picked fruits). The actual time for the change yellow-green to red was approximately the same.

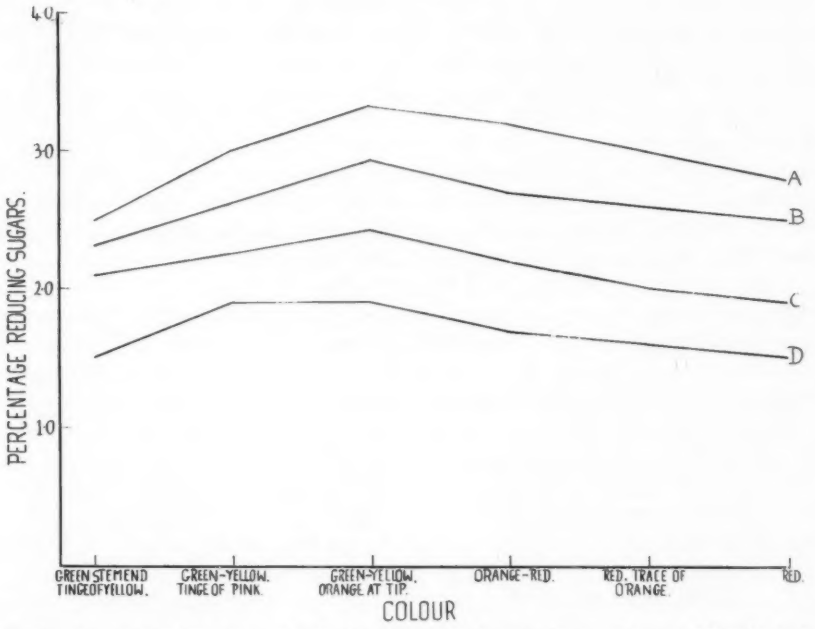

Fig. 8. Curves showing the total reducing sugars during ripening. Curve A "first" fruits, curve B "intermediate" fruits, and curves C and D "last" fruits.

Similar variations in the maximum respiratory intensity were also recorded in this series, i.e. the distinction between "first", "intermediate" and "last" fruits becomes apparent. The peak in prematurely picked fruits is not as high as in late picked fruits, which may be attributed to a loss of turgidity or reduction in the sugar content in the former. Fig. 7 shows two typical curves, the upper of which was obtained from a "first" fruit, and the lower from an "intermediate".

3. *Carbohydrate Content.* Since it was impossible to trace the curve in a single fruit is was necessary to do analyses of a large number of fruits whose positions on the truss were carefully noted. A series was done of "first" fruits

at different colour stages; the points were plotted as a graph representing the course of the reducing sugar content during the ripening stages of "first" fruits (Fig. 8). A similar procedure was followed for "intermediate" fruits, and for "last" fruits. In every case, the general trend is the same, showing a maximum in fruits which are green-yellow with a tinge of orange at the tip.

The starch content of tomato fruits is at a maximum shortly before the appearance of colour. With the appearance of colour, a diminution of the starch content has already commenced, and hydrolysis is complete at the orange-red stage. It would thus be expected to find the peak in the reducing sugar curve at the orange-red stage, or probably slightly before, since the respiratory activity would tend to reduce the sugar content. This theoretical position of the peak corresponds with that found by analysis.

Many more readings for reducing sugars (all of which were high) were plotted, but insufficient were obtained to form a complete series. These high values were associated with fruits which developed alone on the truss ("lone" fruits) and therefore received an abnormal amount of food material. A similar condition may be artificially created by removing all the fruit but one from the truss. The single large fruit was noticeably sweeter than any other.

The respiratory intensity and the percentage of sugars in the fruit are definitely connected, the fact being demonstrated by graphing the respiratory curve and doing an analysis of the same fruit as soon as it reached the red stage. It is important to compare only figures obtained from fruits at the same physiological stage, so that, when a full red colour appeared, the fruit was removed from the respiratory jar and the total reducing sugars were estimated. By this means a most significant fact was established: *that the curves with the highest peaks were associated with the highest sugar content.* Similarly, with low respiratory values, low sugar values were found. Four such curves are to be found in Fig. 5. Curve A was obtained from a "first" fruit whose sugar content was 3·9 p.c., curve B from a "first" fruit with 2·7 p.c. sugar, curve C from an "intermediate" fruit with 2·4 p.c. sugar, and curve D from a "last" fruit with 1·86 p.c. sugar. Table 3 shows the relationship between respiratory intensity and total reducing sugars:

TABLE 3.

Showing the Relationship between Respiratory Intensity and Total Reducing Sugars.

Maximum respiratory intensity. Mg. of CO_2 per 100 gm. of fruit per hour.	Mass. Gm.	Percentage reducing sugars.
8·7	124	3·9
8·5	71	3·05
7·7	84	2·74
7·0	77	2·53
6·3	84	2·43
5·4	66	2·00
5·2	58	1·86

It has been mentioned above that at the end of the truss there is a varying number of tiny fruits which are fertilized last and are extremely slow to develop, reaching a diameter of one or two centimetres at the time when the "first" fruits are mature. The sugar content of these small fruits is extremely low before the removal of the ripe fruits. After the latter are picked, the percentage of reducing sugars rises rapidly, in a few cases reaching 4 p.c. As these fruits ripened very quickly, no detailed analyses could be done.

DISCUSSION.

In the results outlined above, it is quite obvious that the position of the fruit on the plant determines not only the maximum size attained by the fruit, but also its carbohydrate content, respiratory intensity and growth rate.

The maximum size naturally affects the gradient of the growth curve obtained. Also, the rate of increase in size is least in small fruits, which may be attributed to the fact that a fruit which is fertilized late in comparison with the "first" fruit, does not commence rapid growth immediately, but three or four weeks may elapse before the marked increase in volume commences.

Gustafson (1926) states that morphological and physiological age are not identical but may be separated by a considerable time, or in other words, a number of weeks may elapse before the young fruit commences to grow, so that this fruit would be physiologically a number of weeks younger than a fruit which was fertilized at the same time, but which commenced to swell immediately.

It is quite obvious, on examining any truss of fruits, that there is a diminution in the size of the fruit on passing down the truss from the main axis. A careful examination of the truss shows that the flowers are fertilized in quick succession, commencing with the flower nearest the main stem. This "first" fruit commences growth and is followed, but more slowly, by the "intermediate" fruits and more slowly still by the "last" fruits. It seems that the "first" fruit monopolizes the food material and reduces considerably the amount of food passed to the more remote fruits which naturally grow less quickly; but *there is not a cessation of growth* during the very young stages of the "intermediate" and "last" fruits. The growth rate of these fruits during the first few weeks is extremely slow, but with careful observations, slight increases in size were detected.

Sufficient proof that morphological and physiological age are not widely separated is given by the following observations on the tiny fruits borne at the end of the truss. These fruits at the age of ten weeks are about one centimetre in diameter, and when the normal fruits are removed from the plant, there is an influx of food material into them resulting in the appearance of colour. If these fruits were many weeks younger physiologically than the "first" fruits, one would expect to find that they would grow to the normal size and finally ripen. The physiological age of these fruits is thus very near to the morphological age, which is further supported by the fact that the seeds, though small, are mature. These

fruits must therefore be regarded as a distinct physiological type whose maximum size is small and whose growth rate is slow. Similarly in "intermediate" and "last" fruits, physiological and morphological ages are very close since the fruits at maturity have a lower percentage of sugars, a slower growth rate, etc., than the "first" fruits and must therefore not be considered as physiologically younger at the same morphological age, but must be regarded as distinct physiological types.

Further evidence that the food material is monopolized by the "first" fruits is given by "lone" fruits. It is by no means uncommon to find a single normal fruit on a truss, the remaining fruits being very little larger than the ovary. In this case, it appears that the "lone" fruit, being fertilized first, commences growth and reaches the rapid period of swelling before the more remote fruits commence growth. The huge demands of the "lone" fruit incurred during the rapid growing period completely prevent the passage of food material to the other fruits, which remain small and frequently fall. The removal of the "lone" fruit permits a transference of food to the smaller fruits, resulting in swelling.

The differences in the reducing sugar content of the fruits are a natural result of the position of the fruit relative to the food supply, so that "first" fruits have the highest percentage of sugar, and "last" fruits the lowest.

In tomato fruits, the rate of ripening of the different types varies but slightly; nevertheless, the three types outlined above resemble those of Blackman (1928). Since Blackman used a fruit whose natural life is longer than that of the tomato, the difference in the time period for ripening would be more easily detected.

The main point which this study has revealed is that the fruits differ chiefly in their chemical composition which in turn affects not only the rate of ripening, but also the respiratory intensity. Some factor other than the percentage reducing sugars is associated with the respiratory intensity, since respiration is not proportional to the reducing sugar content.

External factors may in part cause the varying sizes of fruits on an individual truss. The maintenance of a large number of fruits requires not only a large leaf surface, but also a favourable temperature, etc. The leaf surface is probably not a limiting factor since plants with approximately the same leaf area grown with artificial heat are capable of maintaining a larger number of fruits than plants grown without extra heat. The low temperature during the winter seems to be the only factor capable of making more apparent the differences in the physiological nature of the fruit. Since these differences occur in fruits grown with internal heat, it seems probable that a low temperature accentuates the variations between the physiological types.

SUMMARY.

The growth of tomato fruits has been investigated from the young to the red-ripe stage and curves of the typical growth nature were obtained. The relationship between the growth rate, respiratory intensity and carbohydrate content was

studied and the fruits have been divided into three main physiological groups depending on the position of the fruit on the truss.

An important relationship exists between the position of the fruit on the truss, the sugar content, respiratory intensity, growth rate and maximum size of the fruit.

The climacteric rise occurs and is coincident with the appearance of colour in the fruit.

The respiratory intensity attained at the peak of the climacteric rise is closely connected with the position of the fruit on the truss. The fruits which ripen first have the highest sugar content, the highest respiratory intensity, and occupy the 'first'' position on the truss.

Premature picking does not prevent the occurrence of the climacteric rise, but the maximum value attained is reduced.

The curve for the percentage of reducing sugars during ripening is followed closely by the climacteric curve, but the maximum is reached slightly before that of the climacteric rise.

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ON THE PRESENCE OF AN "OXIDASE" IN THE JUICE EXPRESSED FROM TOMATO PLANTS INFECTED WITH THE VIRUS OF TOMATO SPOTTED WILT[1]

by

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INTRODUCTION.

From the behaviour of inocula of the virus of tomato spotted wilt (T.S.W.) it was deduced (Best and Samuel, 1936b) that the juice expressed from infective tomato plants contained a catalyst which catalysed the aerobic oxidation of certain phenolic substances. It was also deduced that juice from infected field plants contained in addition to this catalyst a substance which when present in the oxidized state was capable of inactivating the virus of T.S.W. Subsequent work has established the correctness of these deductions. Although this work is not yet complete, certain of the findings are of general importance to the handling and preservation of inocula, to the demonstration of active virus in suspected hosts and to the interpretation of infectivity data. They are accordingly presented here.

EXPERIMENTAL METHODS.

The power of the catalyst to cause a rapid browning of solutions of catechol when exposed to air has been used as a test for its presence. Unless otherwise stated the tests were made by adding one volume of freshly expressed plant juice strained through fine lawn to 24 volumes of composite phthalate-phosphate-borate buffer solution (0·04 M) of pH 7 in which the reagent had been dissolved to give a 0·01 M solution.

Infectivity tests were made by the primary lesion method as described in previous papers and by systemic infection of tomato plants.

When diseased and healthy plants were compared these were taken from the same batch and were therefore comparable with respect to age and environment. Most of the tests were made with plants raised in an insect-proof glasshouse where healthy and artificially infected plants were grown in adjacent compartments. The term "healthy" as applied to plants grown in the field refers to plants which showed no disease symptoms and which yielded a non-infectious juice.

[1] Glasshouse facilities and part of the cost of these investigations are being provided by the Council for Scientific and Industrial Research.

EXPERIMENTAL RESULTS.

When the juice from the tops of tomato plants which had been artificially or naturally infected with T.S.W. virus was added to a solution of catechol and the resultant suspension was exposed to air in a test tube, a brown ring formed at the surface after a few minutes' exposure. After further standing the zone of pigment formation extended downwards until, after some hours, the whole mass was coloured a deep brown. Still longer standing resulted in a darkening of the colour to what appeared to be black by reflected light, but on dilution the brown colour persisted. A similar reaction was given when the catechol was replaced by quinol, phenol or tyrosine (saturated solution of the latter).

The juice from comparable parts of healthy plants, whether raised in the glasshouse or in the field, did not give these reactions. Controls with catechol in buffer alone gave no colour in the times over which the tests extended and (at pH 7 or less) developed only a faint colour after 48 hours. The catechol test has been applied on a large number of occasions extending over a period of three years and similar results have always been obtained.

When oxygen was excluded by bubbling oxygen-free nitrogen or hydrogen through suspensions in specially constructed vessels, no colour developed. The coloured compound in question did not form if cystein, Na_2SO_3, $Na_2S_2O_4$ and certain other reducing agents were also added in sufficient amounts. When such reducing agents were added subsequent to the development of the colour this latter was discharged.

Effect of Heating the Juice and Other Treatments.

Boiled infected juice before or after filtration did not give the reaction. An instantaneous pink colouration was produced throughout the solution but this reaction is totally different in nature from the one under discussion.

The supernatant liquid obtained by centrifuging infected juice at a force of 1,500 g. for one hour gave a positive reaction of the same order as an untreated aliquot but after boiling this centrifugate for one minute and then cooling it the test was negative.

The catalyst responsible for the reaction was evidently rendered inactive by boiling for one minute. Because of this and other properties the catalyst may be classed as an enzyme. Throughout this paper the enzyme will be considered as a single entity for the sake of convenience but there is no proof that only one enzyme is involved.

The enzyme was active over that portion of the pH range over which the virus of T.S.W. was active, but at pH 5 the action, as judged by the colour development, falls sharply and at pH 4 it is very feeble. The concentration of electrolyte affected the ultimate colour developed, this being inhibited by 0·1 M NaCl (in the presence of buffer). Infectivity tests have shown that lesion production of T.S.W.

on *N. tabacum* may also be depressed by increasing the salt concentration above about 0·005 M. This does not appear to be due to precipitation of the virus particles because even at 0·2 M concentration of salts, the virus remains in stable suspension when centrifuged at 1,500 g. and is only precipitated by ammonium sulphate at concentrations of 20 p.c. (slowly) and 25 p.c. (more rapidly).

The enzyme is quite active in infected juice which has been exposed to air at room temperature for 24 hours and which contains no active virus.

Occurrence and Distribution of the Enzyme within the Plant.

The varieties of *Lycopersicum esculentum* with which the author has worked are Dwarf Champion, Early Dwarf Red, Sensation, and a hybrid between Early Dwarf Red and Break o'Day. The leaf juice of all these varieties, when infected, contains the enzyme.

During the normal growing season the occurrence of healthy virus-free plants in the field is open to considerable doubt on account of the high percentage of plants which ultimately become infected. However, late plantings towards the end of the 1935 summer resulted in a field crop which in late autumn were vigorous and only 50 p.c. of the plants were found to be naturally infected by T.S.W. The remaining plants were free of all disease symptoms and no virus could be demonstrated in them by inoculation experiments with expressed juice from a number tested. Juice from these healthy field plants gave no reaction whatever with the catechol reagent, whereas juice from the infected plants gave strong positive tests.

Table 1 illustrates the distribution of the enzyme within a single diseased plant.

TABLE 1.

Showing the Presence of an "Oxidase" in Various Parts of a Long Diseased Tomato Plant Infected with the Virus of Tomato Spotted Wilt.

	Relative intensity of reaction with catechol reagent. Time after mixing.	
Part of plant.	2½ hours.	4½ hours.
Well bronzed young leaflets	+	++
Stems at top	+	++
Large leaves once slightly affected but recovered	−	−
Main stem	++	+++
Roots	+++	+++++
Healthy control (leaves)	−	−

The results of a typical series of tests with juice derived from various sources are summarized in Table 2.

TABLE 2.

Showing the Relative Intensity of Pigment Formation by Tomato Plant Juice (Tops) from Various Sources, in the Presence of Catechol and Buffer Solution of pH 7.

Source of Juice.	Relative Intensity of Colour Formation in the Catechol Test. 5 mins. after mixing.	80 mins. after mixing.
Healthy *L. pimpinellifolium* tops (glasshouse)	–	–
Do. (field)	–	–
Healthy Dwarf Champion tops (glasshouse)	–	–
Dwarf Champion tops artificially infected (glasshouse)	++	+++
Do. naturally infected in the field	+	+++
Healthy hybrid (Early Dwarf Red x Break o' Day) tops (glasshouse)	–	–
Do. artificially infected with T.S.W.	++	+++
Do. field plant, showing symptoms of T.S.W.	++	+++

The greater the rate of appearance of the colour, the deeper was the final colour developed over a 24 hour period.

In another experiment comparative tests were carried out with catechol and tyrosine on juice from various parts of healthy and artificially infected glasshouse plants (var. Dwarf Champion) at the same stage of development. The results are recorded in Table 3.

TABLE 3.

Reaction of Juice from Various Parts of Healthy and Diseased (T.S.W.) Tomato Plant to (A) Solutions of 0·01 M Catechol in Buffer Solution of pH 7 and (B) Saturated Solutions of Tyrosine[2] in Buffer of pH 7.

Time after mixing.	Tops. Spotted Wilt.	Tops. Healthy.	Stems. Spotted Wilt.	Stems. Healthy.	Roots. Spotted Wilt.	Roots. Healthy.
A. *Catechol.*						
15 mins.	+	–	++	?	+	?
2 hours	++	–	++++	?	++	+
6 hours	++++	–	++++++	?	++++++	++++++
B. *Tyrosine.*						
5 mins.	–	–	?	Not	–	–
1½ hours	+	–	+	deter-	+	+
24 hours	++++	–	++	mined	+++++++	+++++++

[2] The tyrosine was a very pure sample kindly supplied by the Animal Nutrition Laboratory, Council for Scientific and Industrial Research.

Tobacco Leaves.

Juice from tobacco leaves (var. Blue Pryor) carrying primary lesions of tomato spotted wilt gave a positive catechol test whereas juice from leaves of healthy plants of comparable age did not.

Nasturtium Leaves.

The symptom picture of nasturtium leaves infected with tomato spotted wilt is very different from that of either tomato or tobacco plants. A variety of symptoms are shown but the main symptoms are of the nature of a mosaic of dark and light green areas and a total absence of pigmentation; although ring spot symptoms develop on some leaves it is unusual even then to get pigmented lesions. Necrotic response is also rare. The plants do not appear to suffer any serious consequence as a result of infection except when heavily infected in the seedling stage. It was therefore of some interest to test juice from these plants for the presence of the enzyme. The results of such tests are recorded in Table 4.

TABLE 4.

Catechol Test (at pH 7) for the Presence of Oxidase in the Leaf Juice of Nasturtium Plants (var. Golden Gleam) Artificially Infected with Tomato Spotted Wilt Virus. The Results of Parallel Tests with Leaf Juice of Infected Tomato Plants are Recorded for Comparative Purposes.

Time after mixing.	Nasturtium. Healthy.	Nasturtium. Spotted Wilt [Juice] = 1/10	Nasturtium. Spotted Wilt [Juice] = 1/25	Tomato. Spotted Wilt [Juice] = 1/10
5 mins.	−	−	−	−
3½ hours	−	−	−	+
9½ hours	−	−	−	++
21½ hours	−	−	?	++++
70 hours	?	?	++++	+++++++++

The enzyme appears to be absent from the leaf juice of nasturtium plants whether healthy or infected with the virus of tomato spotted wilt. There is, however, the possibility that it is present but that reducing conditions in the juice prevent it taking part in the oxidative action. Discussion of the bearing of these results on the plant-virus interaction and on the symptom picture may be conveniently postponed until a later paper when further results will be presented and discussed.

Effect of Enzymic Oxidation of Phenols on Virus Activity.

The action of phenolic substances, quinones and hydroxy-quinones on the activity of T.S.W. virus will form the subject of a future paper. Certain results,

however, are relevant to the present discussion. Substances such as quinol, phenol and catechol inactivate the virus within a few minutes when exposed to air but do not do so if air is excluded, nor do they do so if cystein or sodium sulphite is also present. In the presence of these substances (phenols) and air, redox potentials are developed which fall on the positive side of the critical range for this virus. It may therefore be concluded that tyrosinase catalyses the oxidation of these substances by air and that the oxidation products then inactivate the virus.

Presence of Autoxidizable Substance in Juice of Infected Plants.

When the juice expressed from the tops of infected field tomato plants was exposed to air as a suspension in buffer solution of pH 7, it rapidly became brown. This browning was more marked at some times than at others and the rate of inactivation of the virus in such suspensions ran parallel with the rate of browning. When suspensions of the freshly-expressed juice were maintained in an atmosphere free from oxygen, this browning did not take place (occasionally perceptible browning occurred during crushing and transfer to the oxygen-free vessel, in which instances the resultant suspension had a brown tinge but the colour did not deepen subsequently, and often became perceptibly lighter). However, when aliquots of suspensions stored in this way for some hours were removed and exposed to air they rapidly became brown and the virus became inactivated at a rate comparable with colour development. After a short time such aliquots were completely inactive whereas the suspension from which the aliquot was drawn was almost as active as before. The colour development and inactivation of the virus were both arrested by the addition of those reducing agents which normally preserve the virus against oxidative inactivation. The colour, once developed, was discharged by the same reducing agents, but virus, once inactivated, was not reactivated by this treatment.

Suspensions of juice from healthy plants remained light green under the same conditions. Infected plants raised in the glasshouse behaved in a manner intermediate between that of healthy plants and infected field plants. Suspensions of the leaf juice of infected glasshouse plants never developed a deep brown colour, frequently remained light green often with a tinge of yellow, and occasionally developed a very light brown colour. The general behaviour of those suspensions which became brown was similar to that of suspensions of infected field plant juice.

It is interesting to note that the normal inactivation of virus in suspensions exposed to air, proceeds less rapidly when the plants or plant material (kept in water under a bell jar) from which the juice is expressed are stored in the subdued light of the laboratory for a full day before being used. Furthermore such suspensions do not become brown as rapidly as suspensions prepared from material freshly brought from the field. It appears that the substance responsible for the browning and associated processes is present in greatest amount during active photosynthesis and is depleted when respiration exceeds photosynthesis.

Oxygen Uptake of Juice from Healthy and Diseased Plants.

The rate of oxygen uptake as measured in a Barcroft manometric apparatus[3] was significantly greater for juice expressed from tomato plants infected with T.S.W. than for juice from healthy plants of the same age.

From this and the preceding section it is clear that juice from infected field plants contains an autoxidizable substance capable, when in the oxidized state, of inactivating the virus and that the juice of infected glasshouse plants also contains the substance but in much smaller amounts.

Peroxidase in Leaves of Healthy Tomato and Tobacco Plants.

Leaflets of healthy tomato plants and leaves of healthy tobacco plants were ground in a mortar and thoroughly extracted with 95 p.c. alcohol. The white residues were further ground in the presence of water and filtered. The turbid filtrates gave, in both cases, a strong positive test for peroxidase[4] (guaiacum + H_2O_2). When freshly prepared these preparations gave a weak "oxygenase" or direct oxidase test but no longer did so after standing overnight.

Leaflets of tomato plants infected with T.S.W. when treated as described above behaved similarly.

Addition of these crude peroxidase concentrates to a solution of catechol in buffer solution of pH 7 did not cause the production of brown pigment, so that the peroxidase could not have been responsible for the reactions described in previous sections.

The fluorescent substance previously described (Best, 1936) did not give the catechol reaction.

DISCUSSION.

Leaves of healthy tomato plants do not contain sufficient tyrosinase to give a positive test under the conditions of these experiments, so that, if present, it must be so in very small amount. Leaves of tomato plants affected by T.S.W. contain relatively large amounts of the enzyme. The question arises as to whether the enzyme present in diseased leaves was produced *in situ* as a result of the virus-plant interaction or whether breakdown of phloem tissues along the path of the invading virus enables the enzyme to pass from the roots to those portions of the plant invaded by the virus. Samuel (1934) has shown that the path of tobacco mosaic virus in a tomato plant is from the inoculated leaf down to the roots and from there up to the rest of the plant. Caldwell (1930, 1931) has demonstrated that this virus moved only in the phloem elements. Unpublished observations of the author indicate that T.S.W. virus moves in the vascular bundles of the tomato plant and

[3] I am indebted to Professor Sir Stanton Hicks for the loan of this apparatus.

[4] This is contrary to the findings of Tschailachian and Alexandrovskaja (1935) who state that tomato leaflets do not contain peroxidase.

since the pH value of the xylem contents is such that this virus would be inactivated there, it is reasonable to conclude that this virus also moves[5] in the phloem elements. If the path of the virus from the inoculated leaves is similar to that found by Samuel for tobacco mosaic, then the chances of some of the root contents passing to those parts of the rest of the plant invaded by the virus are very high. However, by whatever agencies the increase in the enzyme content of the leaves is brought about, it is quite likely that an excess of tyrosinase in the leaves may actually be the cause of part at least of the damage and pigmentation, especially when a certain amount of disorganization throws enzyme and substrates into closer contact than usual. It is possible that this is also one of the causes of the necrotic response in tomatoes as contrasted with the (usually) non-necrotic response in plants like nasturtium.

On account of the formation of a brown oxidation product in the juice expressed from infected field grown plants and to a lesser extent in juice expressed from infected glasshouse plants, it is essential to crush out the juice in the presence of a buffered solution of some suitable reducing agent when the concentrations of virus in different plants or different parts of the same plant are being compared. The volume of reducing solution added is known and the total volume of added solution plus juice may be measured and made up to some standard juice concentration.

This procedure is also important when testing the juice from a suspected new host for T.S.W. virus, to avoid possible inactivation by oxidative processes, and has been used by the author for this purpose for some years past. However, as previously pointed out (Best and Samuel, 1936a) it is of equal or greater importance to insure that the pH value of the expressed juice is buffered at a pH value at which the virus is known to remain active. Failure to recognize and take these precautions may easily result in erroneous conclusions. The use of a reducing agent for this purpose has already been recognized by Ainsworth (1936) when dealing with T.S.W. in chrysanthemum juice although it is not known how much of the preservative effect obtained by him was due to changes in pH value (which were not known) and how much to reducing conditions. It is very likely that some of the other less resistant viruses may be similarly affected and that similar precautions should be taken.

When rates of inactivation of T.S.W. inocula in the absence of oxygen are being measured it is necessary to protect aliquots, which are drawn off at suitable time intervals, from aerobic inactivation during the inoculation procedure. Practical difficulties prevent the total exclusion of oxygen during the actual inoculation process, but this condition may be approached by transferring these aliquots

[5] The term "moves" is used in the sense of direction of propagation. It is not known how much of virus "movement" is due to transport of individual particles and how much through propagation dependent on a series of chain reactions to which actual movement of virus particles is not essential.

at once to stemless dropping funnels through which a stream of oxygen free nitrogen or hydrogen is passing. The inoculum may then be applied to the spatula a few drops at a time as required for each leaf. For many experiments the most satisfactory procedure is to run an aliquot of an inoculum into a known volume of a solution of a suitable reducing agent already pipetted into the funnel. In this way any further action is arrested.

SUMMARY AND CONCLUSIONS.

It has been shown that the juice expressed from the leaves of tomato plants infected with the virus of tomato spotted wilt contains an "oxidase" enzyme (tentatively identified as tyrosinase) which catalyses the oxidation of phenol, catechol, quinol and tyrosine in the presence of air. The reaction does not proceed to a demonstrable extent in suspensions of juice expressed from comparable parts of healthy plants but it does in suspensions of root juice.

The enzyme was also present in juice expressed from tobacco leaves infected with T.S.W. but not in the juice from similarly infected nasturtium leaves.

The juice of infected field-grown tomato plants was found to contain in addition an autoxidizable substance whose oxidized form was capable of inactivating the virus *in vitro* in suspensions buffered at pH 7. It was concluded that the rapid oxidation of this autoxidizable substance in air is catalyzed by the enzyme and that the oxidized form of the substance inactivates the virus by direct oxidation.

The rapid inactivation of T.S.W. virus in suspensions containing phenol, catechol or quinol exposed to air received a similar explanation. In the presence of free oxygen the enzyme catalyses the oxidation of the phenols and the oxidation products then inactivate the virus.

The bearing of these results on some plant-virus inter-relationships and on the handling of suspensions of the virus, is discussed.

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CREATINE RETENTION IN EXPERIMENTAL RENAL INSUFFICIENCY

by

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Recently a new method for the determination of creatinine was described almost simultaneously by several independent workers (Bolliger, 1936; Benedict and Behre, 1936; Langley and Evans, 1936). This method was then also applied to the determination of creatine, and since a review of the literature revealed little information with regard to creatine retention in renal insufficiency, it was decided to investigate this problem further. At this juncture, four dogs became available which had been used in another investigation and which were acutely uraemic. The blood of these animals was examined for its creatine content. The results obtained were somewhat unexpected, and differed from those obtained in clinical cases. They are, therefore, being reported separately.

TECHNIQUE.

Experimental Animals.

The dogs at our disposal had one kidney implanted into the omentum after which the renal vessels were cut. The other kidney was removed before or after cutting the vessel of the implanted kidney. In one instance the remaining kidney was destroyed completely by spontaneous hydronephrosis. In all four cases, acute uraemia due to anuria was present at the time the post-operative blood specimens were taken. Blood specimens were obtained from the saphenous vein, and examined for creatinine, creatine, and urea.

Preparation of Filtrates.

In normal dogs trichloracetic filtrates were made from whole blood and plasma by adding a half volume of 50 p.c. trichloracetic acid to each volume of plasma or whole blood. These filtrates were neutralized with 20 p.c. sodium hydroxide, using litmus paper as indicator. In the uraemic dogs, tungstic acid filtrates were prepared by adding to one volume of blood, plasma or cells, three volumes of a solution containing 1·5 p.c. anhydrous sodium sulphate and 1·4 p.c. sodium tungstate. After standing for 15 minutes, one volume of 2/3 N sulphuric acid was added. The creatine content of cells in normal dogs was estimated on similar filtrates. These filtrates were found to be sufficiently neutral in reaction without requiring any further correction for any of the subsequent determinations.

Determination of Preformed Creatinine.

Creatinine was determined by the method of Folin (1914) and also by the new dinitro-benzoic acid method (Bolliger, 1936).

Determination of Creatine.

Creatine present in blood filtrates was converted into creatinine by acid hydrolysis under pressure. To 5 c.c. of filtrate was added 0.4 c.c. of 20 p.c. hydrochloric acid. This was autoclaved at 10 lb. pressure for 20 minutes. The mixture was then neutralized with sodium hydroxide, using litmus paper as indicator. After being made up to a convenient volume, the total creatinine content was determined both by Folin's picric acid method and the dinitrobenzoic acid method. The creatine, expressed as creatinine, was obtained by subtracting the preformed creatinine from the total creatinine. In order to convert these results into creatine, the findings have to be multiplied by 1·15.

The Dinitrobenzoic Acid Method for Determination of Creatinine.

Two c.c. of the neutralized filtrate are transferred to a test-tube and 0.4 c.c. of 10 p.c. solution of 3–5 dinitro-sodium benzoate and 0·4 c.c. of N sodium hydroxide are added. In another test-tube, 2 c.c. of the standard solution are treated in the same manner simultaneously. After letting the two solutions stand for 10 minutes, compare them in a colorimeter. The colour developed is a purplish red. As pointed out previously (Bolliger, 1936), the creatinine standards have to agree with the unknown within about 50 p.c. in order to get a reliable colour comparison.

DISCUSSION.

Normal Animals.

The preformed creatinine content of the blood of the dog, as shown in Table 1 is less than 1 mg. p.c. when measured by the dinitro-benzoic acid method. With Folin's method it is somewhat higher, ranging from 0·8 to 1·5 mg. p.c. The creatinine content of cells and plasma is approximately equal. The creatine content of the plasma expressed as creatinine is not more than 0·5 mg. p.c. according to the dinitro-benzoic acid method. The creatine content of the cells is somewhat higher than that of the plasma, about 0·4 — 2·0 mg. p.c. With Folin's method, cell creatine values as high as 2·5 mg. p.c. were obtained. However, it is difficult to obtain values for normal cells and plasma from dogs with the dinitrobenzoic acid method as used by us (Bolliger, 1936), because very concentrated filtrates have to be prepared. On the other hand, the results obtained by Folin's method are open to the criticism which has been levelled against such determinations (Langley and Evans, 1936). Although the results for normal animals are not as accurate as could be desired, this is of little consequence when compared with the findings on uraemic animals.

Uraemic Animals.

The blood chemistry findings on these dogs prior to operation were normal. The results obtained in the four animals after operation are listed in Table 2. They all showed marked preformed creatinine retention in accordance with the clinical

condition, viz. complete anuria. In general, creatinine retention was approximately equally distributed between cells and plasma and there is no marked departure from the relative distribution as found in the normal animal.

TABLE 1.

Creatinine and Creatine Findings in Normal Dogs by the Dinitro-Benzoate Method Expressed in milligrams per cent.

Dog No.	Material.	Preformed Creatinine.	Total Creatinine.	Creatine expressed as Creatinine.
1	Plasma	0·4	0·6	0·2
	Cells	0·5	2·0	1·5
2	Plasma	0·7	1·2	0·5
	Cells	0·7	2·7	2·0
3	Plasma	0·5	0·6	0·1
	Cells	0·5	0·9	0·4

In all cases there was also marked creatine retention. The distribution, however, is an unequal one, since the creatine is retained to a much higher degree in the plasma than in the cells. In the four animals investigated, the creatine retention in the plasma expressed as creatinine varied on the day before death from 17 mg. p.c. to 29 mg. p.c. In the cells it varied from 4 mg. p.c. to 8 mg. p.c. In addition, in Dog 4 as much as 40 mg. p.c. of creatine were found in the plasma of post mortem blood, while the cells contained about 8 mg. p.c. The creatine content of its plasma rose steeply during the two days before death from 10 to 29 mg. p.c. while the creatinine content changed only from 7·6 to 12 mg. p.c. This dog was a young animal, and its uraemia was characterized by marked twitching.

TABLE 2.

Creatinine and Creatine Findings Expressed in milligrams per cent. in Anuric Dogs.

		Preformed Creatinine.		Total Creatinine.		Creatine expressed as Creatinine.			
Dog No.	Material.	Dinitro-benzoate Method.	Pierate Method.	Dinitro-benzoate Method.	Pierate Method.	Dinitro-benzoate Method	Pierate Method.	Blood Urea mg. p.c.	Date.
1	Plasma	14		37		23		580	
	Cells	12		16		4			
	Blood	13		29		16			
2	Plasma	13	12	30	30	17	18	462	
	Cells	12	12	17		5			
	Blood	12	12	26	27	14	15		
3	Plasma	13	13	37	37	24	24	383	
	Cells	14	14	22	22	8	8		
	Blood	14	14	30	30	16	16		
4	Plasma	7·6		18		10		390	26.5.37
	Plasma	12		41		29			27.5.37
	Plasma	13		53	55	40			28.5.37
	Cells	12		20	22	8			28.5.37 P.M.

The smallest creatine retention was obtained in Dog 2, which showed only 17 mg. p.c. in the plasma and about 5 mg. p.c. in the cells. This particular dog, however, had a spontaneous hydronephrosis and died of uraemia complicated by peritonitis subsequent to the last operation. This may explain the comparatively low figure for creatine retention.

The results obtained by the dinitro-benzoate and by the picrate methods agree well when dealing with high values, and this is considered to lend support to the fact that the increase of colour as measured by both methods is probably creatine or some closely related substance or substances.

CONCLUSION.

Marked creatine retention occurs in experimental uraemia, as produced in the dog. The increase in creatine in these cases is much greater in the plasma than in the cells.

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THE SALT AND WATER METABOLISM OF NEPHRECTOMIZED RABBITS

2. THE EFFECT OF INJECTION OF SODIUM CHLORIDE AND BICARBONATE

by

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(Submitted for publication 21st July, 1937.)

INTRODUCTION.

In a previous paper (O'Connor, 1937) a method was described in which nephrectomized rabbits were used to study the extra-renal factors in salt and water metabolism. There it was shown that plasma sodium and chloride fall as the result of the injection of water or glucose solutions, but return to normal within two hours.

Administration of sodium chloride to the intact animal causes changes in plasma chloride which cannot be explained by the urinary excretion. The plasma chloride rises following the ingestion of the salt, but returns to normal while there is still excess salt in the body and while the urinary chloride is still high (Arden, 1934; Melli and Tasso, 1930). Hence it was decided to study the effects of the injection of sodium salts into the nephrectomized rabbit.

PROCEDURE AND METHODS.

The procedure was identical with that of the earlier paper (O'Connor, 1937). Rabbits of either sex, fed on the usual diet of green feed with no additional water, were starved for 24 hours prior to the experiment. Eighteen hours before the experiment both kidneys were removed and the animal allowed to recover. The injection was made into an ear vein over a period of about five minutes. In each instance the salts to be injected were dissolved in 3 c.c. of water per kilogram of body weight of the animal. Blood samples were taken from the ear vein and plasma separated under paraffin for analysis of sodium and chloride content. One sample was taken just before the injection, a second within ten minutes from the completion of the injection, and a third two hours after the injection. The total solid content of whole blood was also estimated and the respiration was recorded as in the previous experiments.

Thus the total solid content of whole blood and the sodium and chloride content of plasma were observed for two hours following the intravenous injection of sodium salts into unanaesthetized rabbits, nephrectomized about 18 hours prior to the experiment.

RESULTS.

Three sets of experiments were performed in which the following salts were injected, the amounts being given in milligram-equivalents per kilogram of body weight of the animal:

Series I. Sodium Chloride, 5·1.
Series II. Sodium Chloride, 4·3; Sodium Bicarbonate, 0·8.
Series III. Sodium Chloride, 3·5; Sodium Bicarbonate, 1·6.

Thus in each case the same amount of sodium was injected, this being equivalent to 2·9 c.c. of 10 p.c. sodium chloride per kilo. The proportion of chloride to bicarbonate was varied.

Controls. Since the methods were unchanged, the control experiments of the previous paper (O'Connor, 1937) were accepted for this series also.

TABLE 1.

Plasma Sodium and Chloride.

Series and Nature of Injected Salts.			Exp.	Plasma Sodium. 0	1	2	Plasma Chloride. 0	1	2
I	Na	5·1	41	12·8	16·8	15·7	9·7	12·3	11·8
			54	—	14·3	14·7	—	10·7	10·9
	Cl.	5·1	55	—	—	—	10·3	11·2	11·0
			56	13·7	15·5	16·0	10·7	—	11·3
			57	13·4	15·6	15·9	9·7	11·7	11·4
II	Na	5·1	42	12·1	14·3	13·0	9·0	10·7	10·0
	Cl.	4·3	52	13·8	16·0	15·2	9·6	10·9	10·8
	HCO_3	0·8	53	12·8	14·7	13·9	8·7	9·9	10·0
III	Na	5·1	58	13·9	15·1	13·9	10·3	10·9	11·2
	Cl.	3·5	59	14·5	15·4	14·3	10·2	11·1	11·3
	HCO_3	1·6	60	13·9	15·7	14·5	11·3	12·2	11·9

The figures for the salt content of the injection are in milli-equivs. per kilo. The plasma sodium and chloride figures are in milli-equivs. per 100 gm. water.

Samples are grouped into 3 time periods:
0 Before injection.
1 Within 15 minutes after injection.
2 Two hours after injection.

Plasma Sodium (Table 1 and Fig. 1a). Immediately after the injection the plasma sodium was raised in all three series. In Series II and III the plasma sodium fell towards its original value during the next two hours, the recovery being more complete in Series III than in Series II. Where the injected sodium was wholly in the form of chloride (Series I) there was no evidence of any return of the plasma sodium to its original level. In fact, the figures actually showed a further small rise during the two hours following the injection. This difference in the response of the three series is well shown in Fig. 1a, where the plasma sodium

figures two hours after the injection fall into definite groups. Where sodium chloride alone was injected the figure remained high, where about 16 p.c. of the injected sodium was in the form of bicarbonate (Series II), there was a lower final value; while where 32 p.c. of the sodium was injected as bicarbonate, an almost complete return to normal occurred.

Plasma Chloride (Table 1 and Fig. 1b). The plasma chloride rose following the injection but showed no similar tendency to return to its original value. There was no difference between the series in this respect.

TABLE 2.

Blood Volume and Calculated Blood Volume.

Series and Nature of Injected Salts.			Exp.	Blood Solids. 0	1	2	Blood Volume. 0	1	2
I	Na	5·1	41	18·4	15·8	17·6	55	62	53
			54	17·4	15·7	15·7	55	59	58
			55	17·5	15·8	16·2	55	60	57
			56	16·4	14·7	15·1	55	59	56
	Cl.	5·1	57	17·9	16·1	15·6	55	60	60
			61*	17·3	15·6	15·7	55	62	61
			62*	18·6	16·6	17·8	55	61	57
II	Na	5·1	42	18·5	17·5	17·7	55	56	53
	Cl.	4·3	52	19·5	17·5	16·9	55	60	60
	HCO_3	0·8	53	18·3	17·5	17·1	55	59	58
			58	16·1	14·7	15·5	55	59	54
III	Na	5·1	59	18·1	16·0	15·3	55	60	61
	Cl.	3·5	60	16·9	15·3	15·4	55	59	56
	HCO_3	1·6	63*	16·2	15·2	15·0	55	59	60
			64*	15·6	14·3	14·6	55	60	59

* In Experiments 61–64 blood solids only were estimated.

The figures for salt content of the injected fluid are in milli-equivs. per kilo. Blood solids are in percentage by weight and blood volume in c.c. per kilo.

Samples are grouped into 3 time periods:

0 Before injection.
1 Within 15 minutes after injection.
2 Two hours after injection.

From the increase in plasma sodium and chloride a few minutes after the injection, may be calculated the volume of fluid in which the injected ions have become diluted. This figure is of the order of 300 c.c. If calculated from the chloride ion, this figure is larger than if calculated from sodium, presumably because chloride is able to enter the red blood cells, whereas sodium cannot. The value of 300 c.c. corresponds to the accepted figures for the volume of extracellular fluid, and so is in accordance with the views of Harrison, Darrow and Yannet (1936), who believe that tissue cells are impermeable to sodium and chloride.

Blood Solids and Blood Volume (Table 2). The blood volume was estimated on the assumption that the total solid content of the blood-stream remained con-

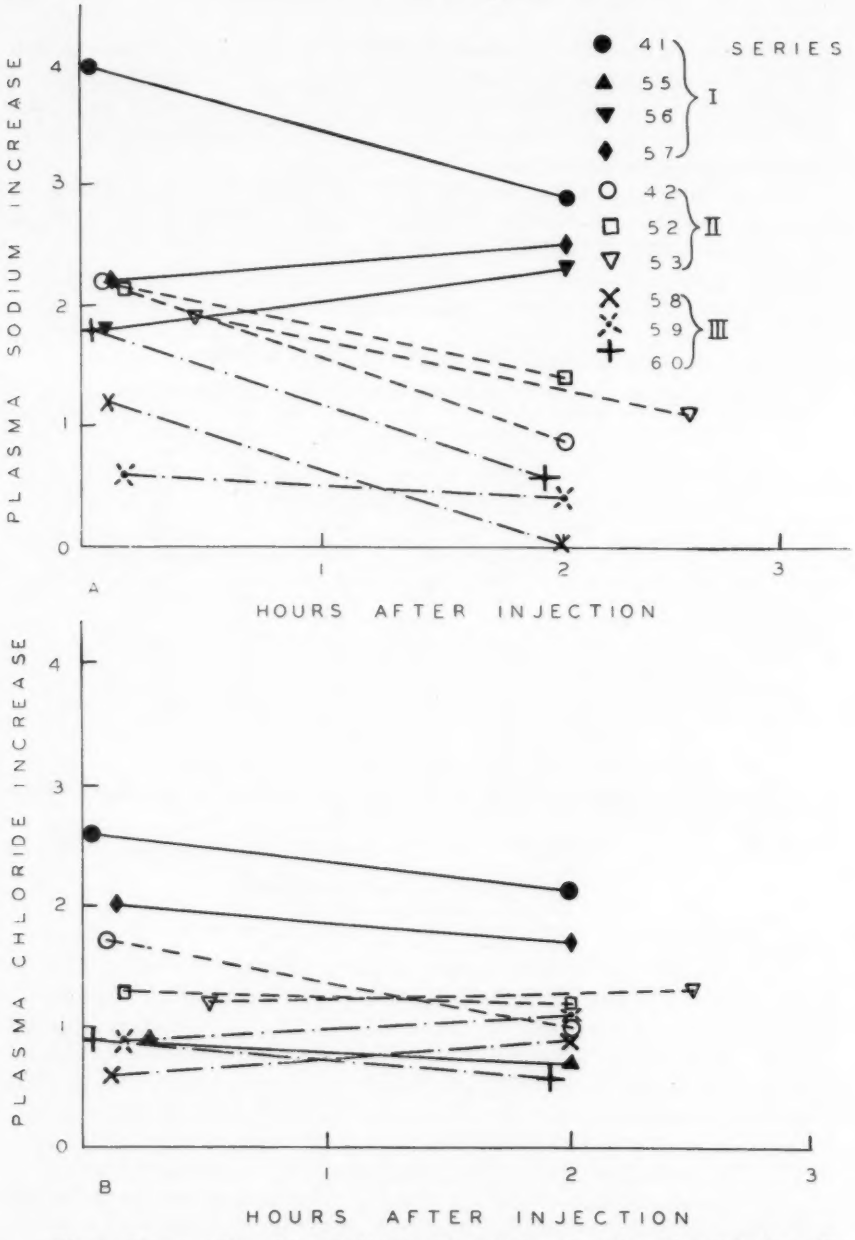

Fig. 1. Plasma sodium (a) and chloride (b) plotted against time after injection. The sodium and chloride figures are plotted as the increase above the figure found before the injection. The unit is milli-equivs. per 100 gm. water.

stant. The calculation was given in detail in the previous paper. In the estimation made soon after the injection there was a dilution of total solids, and consequently an increase of about 5 c.c. per kilo in the blood volume. These changes remained constant, or decreased during the succeeding two hours. Since these changes were small and barely beyond the changes observed in controls, experiments were performed in which only blood solid estimations were made. The above observations were thus confirmed in experiments in which the effect of haemorrhage was eliminated.

Respiration. In all three series the respiration became deeper and the respiratory volume was increased during the injection. This change persisted for less than thirty minutes following the injection. There was no difference between the three series.

DISCUSSION.

It has been shown (O'Connor, 1937) that the injection of water or glucose solutions into the nephrectomized animal causes sodium ions to enter the blood and, presumably, also the interstitial fluid. In the present work the reverse has been demonstrated. The nephrectomized animal, under certain conditions, is able to restore towards normal the raised plasma sodium which results from the intravenous injection of sodium salts. Accepting, as in the previous paper, that the interstitial fluid is an ultra-filtrate of blood plasma, it must be assumed that the interstitial fluid in these experiments undergoes the same changes in composition as those observed in plasma. Consequently, the restoration towards normal of the plasma sodium cannot be regarded as due to a transference of either sodium or water between plasma and interstitial fluid.

The change must consist, therefore, either of a passage of sodium out of plasma and interstitial fluid, or of a dilution of these fluids by incoming water. Since the plasma chloride does not show a corresponding fall towards normal, dilution of the extra-cellular fluids by incoming water does not provide an explanation. Also, the figures for blood solids and calculated blood volume do not support a process of simple dilution. The increase in blood volume immediately after the injection is probably due to the osmotic effect of the increase in sodium and chloride, to which the cells are believed to be impermeable. Later, the blood volume falls and, although blood solid estimations can give no accurate indication of the volume of the extracellular fluid, one would expect at least a small rise in blood volume if the extracellular fluids were increased sufficiently to restore the blood sodium by simple dilution. Thus, it is concluded that in the nephrectomized rabbit injected sodium ions can be transferred to some site outside the extracellular fluid, and that in this transference sodium may be involved independently of chloride. Since it appears from the work of Harrison, Darrow and Yannet (1936) and Hastings and Eichelberger (1937) that sodium cannot enter the tissue cells, the existence of

a depôt area for sodium must be postulated to account for these results. The same conclusion was reached from the results of the injection of water or glucose solutions into nephrectomized rabbits (O'Connor, 1937).

It is not certain that the stimulus for this removal of sodium is the increased plasma sodium itself. Certainly the nature of the anions can influence this process and a high chloride content of the injected fluid prevents the response. However, even in Series III the fluid was not sufficiently alkaline to depress the respiration, and yet the excess sodium was almost completely removed. In this case it would appear that the excess sodium was of itself the stimulating factor and not an alkalosis resulting from the injection.

In these experiments changes occurred in the sodium content of plasma which were not reflected by the chloride, and, therefore, an example is provided of the fallacy of using chloride estimations as an indication of sodium metabolism. It appears that regulation of the cations (mainly sodium) plays an important part in the maintenance of the inorganic composition of the body fluids, and chloride estimations alone can give but little information regarding changes in base distribution.

SUMMARY.

1. The effect of the intravenous injection of sodium chloride and bicarbonate into nephrectomized rabbits has been studied. It was found that the plasma sodium returns towards normal during the two hours following the injection. This change is prevented if chloride without bicarbonate is injected. No similar changes occur in the plasma chloride.

2. It is concluded that the nephrectomized animal is able to remove sodium to some depôt area.

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THE USE OF NUMBERS OF INFECTIONS FOR COMPARING THE CONCENTRATION OF PLANT VIRUS SUSPENSIONS

4. MODIFICATION OF THE SIMPLE DILUTION EQUATION.

by

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INTRODUCTION.

Infection-dilution experiments with purified samples of plant viruses were recently described (Bald, 1937), the results of which were fitted with equations of the form

$$\frac{y}{N} = 1 - e^{-pn_1x}.$$

In this equation y is the number of infections obtained by inoculating on susceptible host plants a virus suspension at a relative concentration x, N is the maximum possible number of infections, p the probability of any one of the n_1x virus particles contained in the suspension entering the plant tissues to cause infection, and n_1 the number of virus particles in an undiluted preparation. Several carefully purified suspensions of viruses gave reasonably good agreement with the equation, and unpurified virus suspensions did not. The difference on the evidence then available was attributed mainly to impurities in the inoculum; but there were other possibilities, since the most carefully purified samples appeared to be less concentrated than the unpurified, and departures from the equation were shown to occur with viruses of the tobacco mosaic group mainly at the higher virus concentrations (Bald, 1937b).

Dilution series have since been published by Stanley (1937a, b) for which the inoculum was obtained by diluting the crystalline protein of ordinary tobacco mosaic and aucuba mosaic, and although the figures for some experiments agree well with the equation, for others they are widely divergent. Some of the series were obtained with concentrated suspensions, so that the maximum numbers of infections (N) were reached, but as the experimental error was not given, it is not

possible to fix the exact range within which N should have fallen. However, a reasonable guess can be made at the magnitude of the error from the numbers of replicate inoculations; generally there were ten for each treatment, and the allowable variation should not have exceeded 50 p.c. except when the numbers of lesions were very few. Divergences greater than 50 p.c. may sometimes be obtained when Stanley's dilution series are fitted with the simple infection-dilution equation. If the protein is pure virus this throws doubt on the suggestion previously made (Bald, 1937b) that distortion of the dilution series is mainly due to the presence of impurities, other than added buffer salts, in the inoculum.

The effects which may reasonably be attributed to the presence in the inoculum of impurities derived from the host plant were demonstrated only at high concentrations of virus and plant juices. They are a rise in numbers of lesions due to better contact between inoculum and plant tissues, and an opposing decrease in numbers of infections due, perhaps, to a destruction of entry points for the virus into the inoculated tissues, perhaps to adsorption of the virus particles on impurities, or to both. The most likely explanation of the long-range fall of infective power, with increasing concentrations of virus, below that calculated from the simple dilution equation now appears to be an aggregation of virus particles.

The method of aggregation will depend on the shape of the virus particle and the whereabouts on it of free bonds. Takahashi and Rawlins (1933) suggested that the particle of tobacco-mosaic virus might be rod-shaped, and Bawden, Pirie, Bernal, and Fankuchen (1936) have recently shown that the active purified protein molecules isolated by Stanley's method have a constant diameter, and are probably more than ten times as long as they are broad. It is postulated that they are "somewhat triangular in shape". At very high concentrations they form liquid crystals in which the fibres are arranged side by side, but not regularly in molecular sheets.

The simplest method of aggregation for rod-shaped particles in solution would be end to end in chains; if aggregation were reversible, dilution would reduce the size and increase the number of aggregates until all were broken up into single particles. The relation between the total number of virus particles and the number of such aggregates in a solution may be formulated as follows:[1]

V_1 = number of virus particles in unit volume of an undiluted suspension.

x = relative concentration of virus particles in a diluted sample.

a_x = number of aggregates consisting of one or more virus particles in unit volume of a suspension at relative concentration x.

r = number of junctions between particles in the aggregates.

At relative concentration x

$$r=\left(\frac{V_1 x}{a_x}-1\right)a_x = V_1 x - a_x$$

[1] This simple solution was kindly suggested by Mr. G. E. Briggs.

According to the law of mass action

$$\frac{dr}{dt} = k_1 (a_x)^2 - k_2 r, \text{ if } k_1 \text{ and } k_2 \text{ are independent of chain length.}$$

At equilibrium

$$r = K_1 (a_x)^2 = V_1 x - a_x, \text{ when } K_1 = \frac{k_1}{k_2}$$
$$K_1 (a_x)^2 + a_x - V_1 x = 0.$$

TABLE 1.

Exp.	Virus.	Inoculum.	Dilution of inoculum $\left(\frac{1}{x}\right)$	Buffer.	Concentration of buffer molarity.	pH value after inoculation.	Lesions observed.	Lesions calculated.	Constants.
1	Streak	Crude juice	20	None			192	197	N = 230
		from tomato	100				113	128	qv_1* = 17·2
		not a single	500				71	61	K = 4·0
		lesion strain	2,500				25	22	
		of virus.	12,500				5·4	5·6	
2a	Streak	Crude, from	50	Potassium	·025	6·2	29·3	28·2	N = 38·4
		tomato, not a	288	phosphate	·0043	6·4	18·1	17·5	qv_1 = 9·33
		single lesion	1,728		·0007	6·5	5·4	6·0	K = 1·072
		strain of	10,368		·00012	6·7	1·2	1·3	
		virus.	62,208		·00002	6·5	·25	·23	
2b	Streak	Crude, from	50	Potassium	·025	6·2	28·1	28·2	
		tomato, not a	288	phosphate	·025	6·2	16·7	17·5	
		single lesion	1,728		·025	6·2	6·3	6·0	do.
		strain of	10,368		·025	6·2	1·4	1·3	
		virus.	62,208		·025	6·2	·15	·23	
3	Streak	Grosser	10	Potassium	·025	6·5	26·9	28·0	N = 30·6
		impurities	25	phosphate		6·9	23·6	23·8	qv_1 = 20·56
		removed with	62·5	phthalate		7·0	17·0	18·0	K = 3·0
		low speed	156			7·0	14·9	12·4	
		centrifuge.	390			7·0	7·0	7·6	
		Single lesion	977			7·0	4·6	4·2	
		strain.	2,427			7·0	2·13	2·05	
			6,070			7·0	·88	0·94	
4	Streak	Centrifuged	40	Potassium	·019	7·0	56·9	59·9	N = 162·5
		at ca 2,000	80	phosphate		7·0	42·3	42·6	qv_1 = 1·53
		revolutions,	160	phthalate		7·0	29·4	29·6	K = 5·0
		single lesion	320			7·1	21·1	18·4	
		strain	640			7·1	10·4	11·1	
			1,280			7·1	6·3	5·9	
			2,560			7·1	3·0	3·1	
			5,120			7·0	1·6	1·7	
5	Streak	Purified but	4	Potassium	·05	7·0	39·5	39·4	N = 42
		pigmented,	8	phosphate			41·1	36·0	qv_1 = 35·2
		single lesion	16	phthalate			26·2	31·0	K = 4·19
		strain.	32				25·1	25·2	
			64				17·8	19·3	
			128				14·2	14·0	
			256				10·5	9·7	
			512				6·2	6·4	

* The value of qv_1 given is for the highest concentration of the inoculum used in each experiment, not for the undiluted plant extract, and x is taken as unity at this concentration.

TABLE 2.

Exp.	Virus.	Inoculum.	Dilution of inoculum $\left(\frac{1}{x}\right)$	Buffer.	Molarity of buffer.	pH value. before inocn.	pH value. after inocn.	Lesions observed.	Lesions calculated.	Constants.
1	Tobacco mosaic	Purified, colourless suspension	1	Potassium phosphate phthalate	·025	7·0	7·0	74·3	73·4	N = 77
			2					68·8	64·7	qv_1 = 5·376
			4					47·6	50·2	K = 0·25
			8					38·4	34·2	
			16					19·6	20·6	
			32					12·0	11·5	
			64					6·1	6·1	
			128					2·74	3·16	
2a	Tobacco mosaic	Crude plant extract	10	None	—	—	—	317·6	318	N = 350
			10^2					184·5	174	qv_1 = 31
			10^3					48·5	54·1	K = 5
			10^4					9·7	9·4	
2b	Tobacco mosaic	Crude plant extract	10^4					10·35	11·33	N = 420
			$\sqrt{10} \times 10^4$					4·04	3·94	qv_1 = ·031
			10^5					1·54	1·29	K = 5
			$\sqrt{10} \times 10^5$					·33	·41	
			10^6					·07	·13	
3a	Tobacco mosaic	Crystalline virus protein purified by Stanley. Solution of protein sedimented by ultracentrifuge	1	Phosphate	·1	7·0		392·0	440	N = 440
			10					455·5	431	qv_1 = 2640
			10^2					276·5	309	K = 17
			10^3					187·0	135	
			10^4					38·7	41·3	
			10^5					9·4	8·8	
3b		30 p.c. of supernatant below upper 50 p.c.	1	Phosphate	·1	7·0		107·2	108·7	N = 440
			10					33·5	31·2	qv_1 = 1·65
			10^2					5·8	6·6	K = 17
			10^3					1·5	0·8	
			10^4					0·1	0·1	
			10^5					0·0	0·0	

In the formulation of the simple equation there was a probability term p (see p. 211) which described the chance of a virus particle *entering and causing infection*; in the present case the probabilities of entering and, having entered, of causing infection will be considered separately.

If q is the probability, when unit volume of inoculum is applied to susceptible tissues, of an aggregate of one or more particles entering the tissues, the equation for the fraction of the inoculum entering may be written

$$K(qa_x)^2 + qa_x - qV_1x = 0, \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where $K = \frac{K_1}{q}$, provided q is the same for aggregates of different sizes.

The probability of one or more virus particles entering the tissues at one point is given by

$$\frac{Y}{N} = 1 - e^{-qa_x} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where Y is the number of points at which entry occurs. In terms of the number of virus particles this may be expressed as

$$\frac{Y}{N} = 1 - e^{-\left(\frac{-1 \pm \sqrt{1 + 4K.qV_1x}}{2K}\right)}$$

If all virus particles entering multiply to cause infection, this is also the probability of infection, but if a proportion is destroyed or rendered ineffective inside the tissues the equation must be modified. The aggregation of the virus molecules is very unstable, and it is a fair assumption that it will not affect the probability of any single particle multiplying to cause infection. Assuming that probability to be the same for all virus particles, if qv_1 is the proportion of the qV_1 particles entering which are effective, and y the number of points at which infections occur, the equation becomes

$$\frac{y}{N} = 1 - e^{-\left(\frac{-1 \pm \sqrt{1 + 4K.qv_1x}}{2K}\right)}$$

The form of the relation is unchanged as the constants K_1, q, and x are unaffected. The result is the same as if a virus suspension of concentration v_1 had been inoculated and all particles entering had been effective. The hypothetical number of aggregates entering to cause infection under such conditions (n_x) will be given by

$$K(qn_x)^2 + qn_x - qv_1x = 0 \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

and the probability of infection by

$$\frac{y}{N} = 1 - e^{-qn_x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

These equations can be used for fitting infection-dilution series. Values of $\log q(V_1x - n_x)$ plotted against $\log qn_x$ will fall about a straight line of slope 2 if they agree with equation 3. By using equation 4, values of qn_x may be calculated (Bald, 1937a) and a value for qv_1 fitted by trial and adjustment. Values of $q(v_1x - n_x)$ may be obtained by subtraction, and a value for K from the straight line of slope 2 which best fits the values of $\log q(v_1x - n_x)$ plotted against $\log qn_x$.

EXPERIMENTS WITH STREAK VIRUS.

Table 1 shows the results of five experiments with streak virus expressed as local lesions given by inoculation of each dilution to leaves of *Nicotiana glutinosa*. The experimental technique has already been described (Bald, 1937a). None of the series agrees with the unmodified theory, so they are fitted according to the modified equation. The constants used, which were obtained by the method described on p. 211 are given in the last column.

Even when unbuffered crude juice is diluted, agreement with the calculated values is obtained (Experiment 1). The greatest divergence is at a dilution of 1 in 500, but it is only 16·4 p.c. of the calculated value, which is well within the experimental error.

In unbuffered juice the concentration of electrolytes varies directly as the virus concentration: this condition is reproduced in Experiment 2, and a comparison is made with the same sample uniformly buffered at all dilutions. The concentration of salts derived from the plant in proportion to those added is too small to affect the comparison. Both dilution series are fitted with the same constants, and the observed and calculated values agree with each other well within the limits of the experimental error. When experiments of the same type were performed with clarified and purified streak virus, the differences in salt concentration again made no difference to the lesion production.[2] The figures for the purified virus, however, were found to agree over the range of concentration tested with the unmodified theory (Bald, 1937a, Table 1, Exp. 3), i.e. little or no aggregation of virus particles had occurred.

Experiments 3, 4 and 5 illustrate results obtained with viruses at three stages of purification; none was pure enough to give a colourless suspension. These experiments were not performed at the same time. The values of K vary from one to five.[3] They represent in part the degree to which the dilution series is distorted, presumably by the aggregation of virus particles, in part the probability of a virus aggregate entering the tissues at one point $\left(K=\frac{K_1}{q}\right)$. Experiments 3 and 4 were the controls at pH 7 for a test of the effects of an acid pH value (4·4) on the dilution series (Bald, 1937b). It was shown that the form of the series at pH 4·4 was different from that at pH 7; it cannot be fitted by means of either the simple or modified dilution equation.

On the other hand Experiment 5 was a control for the effects of inert carbon on the production of local lesions (Bald, 1937c); the series with carbon was of the same form as the series without, showing that if aggregation of virus particles really occurred at high concentrations it was not affected by the carbon, and that the probability, q, of a virus particle entering at any one point was also unaffected. These considerations are concordant with the suggestion that the rôle of inert carbon in increasing the numbers of lesions is purely mechanical (Bald, 1937c).

EXPERIMENTS WITH TOBACCO MOSAIC VIRUS.

In Table 2 are the results of two experiments with tobacco mosaic virus which have already been published (Bald, 1937a, b), and an experiment performed by Stanley (1937b) with crystalline tobacco-mosaic virus protein. Experiment 1 was performed with a colourless suspension of virus, but the results were found not to be entirely in agreement with the simple dilution equation. They may be fitted, however, on the assumption that a small degree of aggregation occurred, with a value of 0·25 for K.

[2] These negative results are not in accordance with the positive effects of electrolytes on the production of lesions by ordinary tobacco mosaic [Samuel, Best and Bald (1935); Thornberry (1935)] but a direct comparison between the two viruses has not been made.

[3] When no aggregation occurs K = 0.

Experiment 2, performed in two parts (a and b) with unpurified plant extract, can be fitted with the same values for the three constants except at the highest dilutions; but in part b the one in ten-thousand and the one in one-hundred-thousand dilutions approach the allowable limit of divergence. However, even if the divergence were counted significant, it might be explained by the inoculation of the two parts of the experiment in succession under slightly different conditions.

The data taken from Stanley's paper (1937b, Table 3, p. 63) represent the dilution of two fractions of a sample of purified tobacco mosaic virus protein which had been centrifuged at high speed. The fractions were the sedimented protein dissolved in 5 c.c. of buffer solution, which was the volume of the original suspension, and 30 p.c. of the supernatant below the top 50 p.c. They can be fitted quite well with the same values of K and N. The values of qv_1 for the two series give an estimate of the relative concentrations of virus in the two suspensions, provided q is the same for both; the suspension of the sedimented portion contains $2{,}640 \div 1{\cdot}65 = 1{,}600$ times as great a concentration of virus as the 30 p.c. of the supernatant.

The value of K is very high, showing great distortion of the dilution series and therefore probably pronounced aggregation at high concentrations of virus, although according to all tests the sample consisted of pure virus supended in ·1 M buffer solution. It is possible that under some conditions impurities may help to prevent aggregation.

EXPERIMENT WITH AUCUBA MOSAIC VIRUS.

Only one example of a dilution series with aucuba mosaic will be given, to illustrate the statement that distortion, which cannot be explained on the aggregation picture, may occur at high concentrations of impurities derived from the diseased plant. Particulars of the experiment are reproduced in Table 3.

TABLE 3.

Inoculum.	Dilution of inoculum $\frac{1}{x}$	Buffer.	Molarity of buffer.	pH value before inocn.	pH value after inocn.	Lesions observed.	Lesions calculated.	Constants.
Crude	2	Potassium	·05	6·8	6·6	80·2	74·9	
plant	4	phosphate		6·8	6·8	113·5	74·4	$N = 75$
juice	8	phthalate		6·9	6·9	83·9	72·4	$qv_1 = 77{\cdot}8$
	16			6·9	6·9	67·6	68·0	$K = 1{\cdot}4$
	32			6·9	6·9	57·9	59·0	
	128			6·9	6·9	39·5	35·6	
	512			6·9	6·9	13·8	15·4	
	2,048			6·9	6·9	4·9	5·0	

At a dilution of 1 in 4 there is a 50 p.c. rise above the maximum, N, which must be assumed to be about 75 if a good fit between the observed and calculated values beyond the 1 in 8 dilution is to be obtained. The rise was previously discussed, and explained by improved contact between the inoculum and the inoculated leaves because of the presence of impurities (Bald, 1937b).

DISCUSSION.

So far most infection-dilution series obtained with viruses of the tobacco mosaic group, if they cannot be fitted by the simple dilution equation, have been fitted within the limits of the experimental error by the modifying equation. This is no proof, however, that the kind of aggregation postulated in the derivation of the equation actually exists, and the constant K must be considered as a measure of distortion of the dilution series rather than a measure of aggregation, unless independent proof is obtained that the virus particles do aggregate in this manner. K may be used as a dependable and rationally derived constant so long as no large body of facts appears to invalidate the equation.

How a change in value of K affects a change in the number of infections may be illustrated by an example. If no distortion occurs y, the number of infections, will approach asymptotically to the maximum as the mean number of particles entering to cause an infection increases. At the other extreme it approaches a relation of proportionality with the concentration of virus particles. When aggregation occurs, provided the conditions of entry into the tissues are the same for aggregates and single particles, there is the same maximum, N, and if dissociation is complete at low concentrations, there is ultimately the same relation of proportionality between y and the relative concentration. At intermediate concentrations aggregation causes the values of y to fall below the values for a solution in which no aggregation occurs.

For any value of K it is possible to find by a graphical method the values of qv_1x at which the values of y obtained with an aggregating solution are, say, 10 p.c. less than the values of y obtained with a solution in which no aggregation occurs. There will be two such values; between them differences greater than 10 p.c. will occur, beyond them differences will be less than 10 p.c. These values are given below for various values of K; also given is the range of relative concentration between them. The range of concentration over which divergence occurs increases much faster than the value of K, the former increases almost as the square of the latter.

K.	qv_1x Max.	Min.	Relative concentration of maximum and minimum.
·125	—	—	—
·25	2·82	·85	3·2
·5	4·73	·34	14
1·0	7·59	·15	51
2·0	13·0	·065	200
4·0	23·4	·031	755
10·0	56·2	·012	4,680

The use of the modifying equation provides a means of obtaining a reasonable estimate of relative concentration of virus from dilution series which cannot be fitted by the simple equation, because a value for qv_1, which is directly proportional to the virus concentration, may be calculated from it. The proportionality

holds, however, only if q remains constant. As an example data were given from Stanley's experiment on tobacco mosaic virus protein, quoted above.

There is a suggestion in some of Stanley's dilution series that one condition postulated in the development of the equation

$$K(qn_x)^2 + qn_x - qv_1x = 0,$$

although it may hold over a sufficient range of virus concentration to make its use legitimate, does not hold at very high concentrations. In some series the values for lesions fall below the maximum, N, with *increase* of concentration, perhaps because the virus aggregates become so big that they have greater difficulty in penetrating entry points in the plant tissues, i.e. q decreases more rapidly than n_x increases and, in effect, N may be reduced in value. As long, however, as this effect is not evident over the lower ranges of concentration, it does not invalidate the use of the equation.

Finally there remain to be explained the apparent discrepancies between the results of experiments, some of which were fitted by the simple dilution equation, and some of which were not. The experiments were performed with material from various sources at different times and by different operators under different conditions, so legitimate comparison of the results is impossible. It remains to be seen if any pre-treatment of the virus, such as crystallization, changes in the electrolyte concentration of the suspensions, temperature, shaking of suspensions to disperse aggregates, and the like can alter the form of the dilution series.

SUMMARY.

An equation, showing the relation between the concentration of free particles and aggregates and the concentration of single virus particles, is developed on the assumption that virus particles in solution aggregate by joining end to end in chains.

The equation is fitted to dilution experiments of streak virus, ordinary tobacco mosaic, and aucuba mosaic which could not be fitted by the simple dilution equation involving no aggregation. Agreement within the limits of the experimental error is obtained in most cases with samples of viruses buffered near the neutral point, and even with unbuffered samples.

The value of the constant, K, which is a measure of departure from the simple dilution law, varies considerably, and, perhaps because the figures for experiments performed at different times under different conditions are fitted, cannot be simply correlated with differences between viruses or any condition of experimentation.

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APPENDIX.

Since this paper was accepted for publication, three others have appeared containing data which bear on the present thesis.[4] In two of them (I, III)[5] independent evidence is given that the particles of tobacco mosaic and related viruses form readily dissociable aggregates by joining end-to-end. However, at high concentrations aggregation is shown also to occur in planes normal to the length of the virus particles, and what form of aggregation exists in more dilute suspensions when the inocula are actually applied to the plant is still unknown.

The other paper (II) contains dilution series for tobacco mosaic and tomato spotted wilt. For purposes of discussion the author divides dilution curves for ordinary and yellow tobacco mosaics into three parts. He writes:

> Section "a" corresponds with high virus concentrations and clearly shows the approach to a limiting number of lesions. . . . This section merges into another, "b", which is essentially a straight line of unit slope. . . . At still higher dilution a change in relative concentration of active virus is accompanied by a smaller change in the number of lesions, giving the section "c".

According to the equation given in this paper there is no discontinuity in the dilution curve, and none but an accidental relation of proportionality between lesions and virus concentration in the section "b".

Changes in the form of the tobacco mosaic dilution series are illustrated (II, Fig. 3, Table 3) by a dilution experiment in which the same inocula were used on two series of *Nicotiana glutinosa* plants differing only in age. As the result of fitting these series by means of the modified equation with the same value of K (2·6), it may tentatively be suggested that the differences were due, first to the production of more possible entry points on the younger plants (N_A, younger, divided by N_B, older, equals 1·44); secondly to a greater percentage survival of the virus particles which entered the tissues of the younger plants (qv_1 was 5 for the younger and 0·5 for the older plants).

[4] I: Best, R. J. (1937): Nature, 139, p. 628. II: Best, R. J. (1937): Austral. J. exp. Biol., 15, p. 65. III: Bernal, J. D. and Fankuchen, I. (1937): Nature, 139, p. 923.

THE PHAGE INACTIVATING AGENT IN EXTRACTS OF *STAPHYLOCOCCUS AUREUS*

by

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It is now a recognized fact that bacteriophages may be inactivated by extracts of susceptible bacteria. Levine and Frisch (1933–34) showed that polysaccharide substances were responsible for the phage inhibition produced by extracts prepared from certain strains of Salmonella and dysentery bacilli. This was confirmed by Gough and Burnet (1934). Both polysaccharide and lipoid fractions prepared from cholera vibrios by Bruce White (1936) contained phage inhibiting substances. The polysaccharide specifically inhibited a phage acting on smooth strains, while the lipoid fraction had an inhibiting action on several phages. Rakieten, Rakieten and Doff (1936) have correlated the susceptibility of staphylococcal strains to lysis by phage with the presence of phage inhibiting agent in their extracts. The work described in this paper is a study of the properties of the phage inhibiting agent (P.I.A.) obtained from *Staphylococcus aureus* extracts.

Preparation of the P.I.A.

The strains of *Staph. aureus* and the bacteriophage used have been described by Burnet and Lush (1935). The strain used for the preparation of the P.I.A. extract was "Eggleston". Au. 2. was the indicator phage. It was grown and titrated on strain "Latham". Extracts of P.I.A. for phage Au. 2. can also be prepared from "Latham".

Overnight grinding of a concentrated emulsion in a ball mill, or treatment with a concentrated solution of urea were convenient methods for breaking up the bacteria. Precipitation of the nucleoprotein with acetic acid removes all the P.I.A. from the supernatant liquid. This precipitate readily dissolves in dilute sodium carbonate solution and the protein can be digested with trypsin, leaving the P.I.A. unimpaired.

The following procedure was employed in the preparation of the P.I.A. extract used for the experiments described in this paper. *Staph. aureus* ("Eggleston") was grown on twelve agar plates of 20 cm. diameter for 48 hours. The bacteria

were washed off with about 8 c.c. of sterile distilled water per plate, centrifuged and re-emulsified in a total volume of 10 c.c. After grinding in the ball mill overnight, the emulsion was poured off and the ball mill washed out with a little water. The combined centrifuged supernatants (20 c.c.) were precipitated with a few drops of 10 p.c. acetic acid, the precipitate collected by centrifugation and dissolved in 15 c.c. of dilute sodium carbonate solution. One c.c. of a crude pancreatic extract was added, the reaction adjusted to pH 8, and digestion allowed to proceed at 37° C. overnight. The digest was precipitated with five volumes of alcohol, and after standing in the refrigerator for two hours, the precipitate was centrifuged down and extracted with 5 c.c. of water. After centrifugation the solution was again precipitated with five volumes of alcohol, to which a little sodium acetate had been added. This precipitate was practically all soluble in water, giving a clear colourless 1 p.c. solution. The yield from this batch was 20 mg. of dried material.

Chemical Properties of the P.I.A.

The material so obtained was an impure polysaccharide. The presence of protein was shown by a positive sulphosalicylic acid test. The amount of precipitate given by this reagent from a 1 p.c. solution of the impure polysaccharide was comparable with that given by serum proteins or egg albumin in a concentration between 0·06— 0·12 p.c. This represents a protein content of about 8 p.c. Control trypsin solutions did not give this precipitate.

Some fractionation of a 1 p.c. solution of the crude material could be effected by alcohol. The addition of one volume of alcohol precipitated a fraction poor in P.I.A. but giving the densest precipitate with sulphosalicylic acid. One-and-a-half volumes of alcohol gave a fraction containing somewhat less precipitable protein, but 70 p.c. of the P.I.A. units. A third fraction obtained with two volumes of alcohol gave the merest trace of precipitate with sulphosalicylic acid, but contained as much (10 p.c.) P.I.A. as the first fraction. Comparative tests of the precipitating power with homologous antibacterial serum and of skin reactivity of the first and second alcohol fractions proved the latter more active in both respects.

The crude P.I.A. gave a positive Molisch reaction and a very faint biuret test. It reduced Fehling's solution after hydrolysis with dilute mineral acid. It gave no colour reaction with iodine. Trypsin and malt diastase have no destructive action on the P.I.A.

Before tryptic digestion of the nucleoprotein the P.I.A. was readily absorbed by aluminium hydroxide C.γ (Willstätter, Kraut and Erbacher, 1925) and by kaolin, and could not be satisfactorily eluted. Filtration through gradocol membranes of relatively high porosity (1μ) resulted in a 75–90 p.c. loss of activity. This was probably due to adsorption. The concentration of the polysaccharide was correspondingly reduced. Filtered extracts were used for all experiments except the chemical tests.

Phage Inhibiting Action.

The filtered solution had a phage inhibiting activity of 100 units. Before filtration it was 1,000 units, as shown in Table 1. This test was carried out as described in previous communications by Burnet (1934) and Burnet and Freeman (1937), allowing serial dilutions of the P.I.A. to react with a standard dilution of phage Au. 2. for four hours at 45° C. 0·02 c.c. of these mixtures was then spread on agar plates previously inoculated with a young broth emulsion of *Staph. aureus* ("Latham") and the number of plaques counted next day. The end point was taken as that of the reciprocal of the concentration of P.I.A. which reduced the number of plaques from the standard dilution of phage to 20 p.c.

TABLE 1.

Titre of P.I.A. Solutions.

		Final dilutions of P.I.A. solutions.				Titre.
		1/2	1/20	1/200	1/2,000	No. of units
1	1 p.c. P.I.A. solution before filtration	1·2	0·8	0·4	36·7	1,000
2	1. filtered through gradocol membrane	0	4·6	36·5	100+	100
3	2. after treatment with anti-bacterial serum	94·7	101	—	—	0

The figures represent the number of plaques, expressed as percentages of the control count, obtained from the mixtures of standard phage dilution with the P.I.A. dilutions shown.

Precipitin Test.

Homologous antibacterial ("Eggleston") serum prepared from rabbits in the usual manner, and having an agglutinating titre to 1 : 12,800, gave a precipitin test to 1 : 100 dilution with this filtered P.I.A. solution (1 in 200,000 dry weight). This test was most rapidly performed as a ring test, using undiluted serum and heating to 45° C. for two hours.

The P.I.A. was specifically precipitated in this test, the supernatant liquid having no activity, as shown in Table 1.

Skin Reaction.

Intradermal inoculation of the material into sensitive human subjects gave a strongly positive skin reaction. A definite zone of erythema appeared in six to eight hours in dilutions as high as 1 : 1,000 of the filtered extract. This persisted for twelve to eighteen hours, and gradually faded. Control tests with trypsin, treated as in the preparation of the P.I.A., were negative.

Action of Dilute Acid and Alkali on the P.I.A.

The phage inhibiting activity is completely destroyed by treatment with dilute acid or alkali at 100° C. Julianelle and Wieghard (1935) prepared polysaccharide extracts from staphylococci by treatment of the bacteria with N/16 hydrochlorie acid on a boiling water bath for fifteen minutes. In high dilutions these substances

gave precipitin tests with antibacterial sera and positive skin reactions. Similar treatment of the polysaccharide described above destroyed all its phage inhibiting activity, reduced its skin activity somewhat, but left its precipitin titre unimpaired. Preparation of crude bacterial extract by this method resulted similarly in a hapten with no P.I.A.

The action of N/10 sodium hydroxide at 100° C. for five minutes also produces a hapten. There is also some reduction in the intensity of the skin and precipitin tests, as shown in Table 2.

TABLE 2.

Comparison of the Properties of the Original P.I.A. and the Hapten Produced from it.

		P.I.A. No. of Units.	Precipitation test. Dilution: 1/2	1/20	1/200	Skin test. Dilution: 1/10	1/100	1/1,000
1	Original filtered extract	100	++	++	+	+++	++	+
2	1. treated N/15 HCl. 15 mins. at 100°C.	0	++	++	+	++	+	±
3	1. treated N/10 NaOH. 5 mins. at 100°C.	0	++	+	±	++	+	±

Readings for precipitin test:

++ precipitate visible to naked eye.
+ precipitate visible with lens.
± trace of precipitate visible with lens.

Readings for skin test:

Largest diameter of erythema—
+++ > 3 cm.
++ 1·5–3 cm.
+ 1·2–1·5 cm.
± about 1 cm.

The diameters of the erythema produced by the acid-treated material were slightly larger than those of the alkali-treated.

DISCUSSION AND SUMMARY.

These results indicate that the same relationship holds between phage and the specific surface polysaccharides of staphylococci as has been demonstrated for Salmonella and dysentery phages by Levine and Frisch (1932–33, 1933–34) and Gough and Burnet (1934), and for some cholera phages by Bruce White (1936). The phage inactivating agent isolated from staphylococci by this method of tryptic digestion followed by alcohol precipitation is impure polysaccharide, which is probably in much the same condition as it exists in the normal bacterial surface. It shows the usual power to precipitate with homologous antibacterial serum and to produce a skin reaction in susceptible persons. Judging from analogy with a similar preparation from *B. aertrycke* studied by Raistrick and Topley (1934), this P.I.A. is probably antigenic, but this has not been tested. By treatment with acid or alkali it loses its phage inhibiting activity, but retains both precipitating power and skin reactivity, thus becoming identical with the hapten prepared by Julianelle and Wieghard (1935).

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THE IMMUNOLOGICAL REACTIONS OF THE FILTERABLE VIRUSES

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THE IMMUNOLOGICAL REACTIONS OF THE FILTERABLE VIRUSES

by

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INTRODUCTION.

We have been led to attempt a general review of the reactions between viruses (including plant viruses and bacteriophages) and their homologous antisera primarily as a result of our work with those viruses which produce discrete pocks on the chorioallantoic membrane of the developing egg. This method, which has recently been described in detail (Burnet, 1936) is particularly adapted to quantitative immunological work with viruses, and we have accumulated a large amount of data in regard to a representative series of viruses. In principle, the method is precisely similar to those used in the quantitative study of bacteriophages (plaque counting methods) and in the recently developed methods with the mosaic viruses of plants, by which the individual foci developing on inoculated leaves are counted. There are both resemblances and differences in the comparative reactions of animal viruses and bacteriophages, and in order to allow an adequate comparison of the two groups, the reaction between a typical bacteriophage (C.16) and its antiserum has been re-studied in detail by two of us (Burnet and Lush). The results of this investigation form the first main section of this review.

The section on plant virus reactions is wholly a compilation from the literature. Of the animal viruses studied by the egg membrane technique in this laboratory, Keogh is responsible for the work with the viruses of vaccinia and Newcastle disease of fowls. The studies on influenza virus have been carried out by Burnet, those on louping ill and infectious laryngotracheitis viruses by Burnet and Lush, and on myxomatosis of rabbits by Lush. The results of some of this work have been previously published, but in all sections a good deal of new work has been incorporated. Although the egg membrane technique has supplied most of the data with which we are immediately concerned, it is recognized that the main interest of virus immunology must always centre on the practical problems of immunity in the naturally susceptible host. An attempt is therefore made to describe and discuss the work of others in which orthodox animal inoculation methods have been used to titrate the infectivity of serum-virus mixtures. Since the majority of such investigations have been made with vaccinia, this virus is discussed more fully than any of the others.

Chapter I.

THE GENERAL NATURE OF VIRUSES AND BACTERIOPHAGES.

Before approaching the main problem, it is advisable to provide a short resumé of the current views (1) on the general nature of viruses and bacteriophages, and (2) on the nature of antibodies and of the antigen-antibody reaction. In regard to the first, we shall adopt the view that viruses and bacteriophages can most usefully be regarded as independent living micro-organisms which are capable of multiplication only within the living cells of the host species. This attitude has been gradually developed by many workers, but has been most explicitly stated by workers at the National Institute for Medical Research, largely as a result of the work of Elford and Barnard on the actual sizes of different viruses. A review of this work was published by Burnet and Andrewes in 1933. Similar studies of the physico-chemical nature of viruses and bacteriophages were also carried out in Bechhold's laboratory in Frankfurt, and the same interpretation has been placed on the results by Bechhold (1934). It can be fairly stated that in regard to all the larger viruses there is now no serious objection to regarding them as living micro-organisms. A considerably greater resistance has had to be overcome in applying the same conclusions to bacteriophages, although the evidence in regard to the large particle phage C. 16 covers exactly the same points as have been established for vaccinia virus. Taking three recent reviews on the nature of bacteriophage activity published in English, Burnet (1934) definitely advocated the biological point of view, regarding bacteriophages as a large and diverse group of independent viruses parasitic on, or living in symbiosis with, bacteria; Krueger (1936) was non-committal, but considered it likely that future work would show bacteriophage to be a non-living protein substance; Topley and Wilson (1936) stress the biological aspects and come to practically the same conclusions as Burnet. Since all the main experimental facts on which the theory of the biological independence of bacteriophages is based have been adequately confirmed, no discussion of the alternative hypotheses will be attempted here.

1. Animal Viruses.

The methods of differential ultrafiltration developed by Elford for the estimation of the particle size of viruses have now been applied to most of the commonly studied types. Table 1, compiled for the most part from data provided by Elford and his collaborators at Hampstead, shows the particle diameter of all the viruses discussed in this review. The single value given is that deduced by Ferry (1936) as being the most probable diameter. In many cases these sizes have been confirmed by other methods such as centrifugation or photomicrography by ultra-

violet light. With one exception, psittacosis, all the results obtained by these methods are consistent with the view that the particles of any given virus are very uniform in size. Omitting the rickettsiae and small cultivable organisms, such as those of bovine pleuropneumonia or the saprophytic strains described by Laidlaw and Elford (1936), all the larger viruses which have been photographed are composed of approximately spherical units. In Barnard's photographs of canary-pox and vaccinia particles, a few oval forms can be seen with a distinct transverse band, probably indicating that multiplication occurs by binary fission. Like most biological materials, virus particles are negatively charged when suspended in fluids at physiological ranges of pH.

TABLE 1.

Sizes of Virus Particles.

Virus	Diameter of particle (mμ)
Psittacosis	300
Vaccinia	175
Canary-pox	175
Herpes	130
Infectious ectromelia	130
Pseudorabies	130
Influenza	100
Newcastle disease	100
Laryngotracheitis	82
Fowl plague	72
Rift valley fever	34
Equine encephalomyelitis	25
Yellow fever	24
Louping ill	17
Foot and Mouth disease	10
Poliomyelitis	10

Direct chemical studies have been made on only one type of virus, vaccinia. Washed elementary bodies subjected to micro-chemical analysis by Hughes, Parker and Rivers (1935) were found to consist largely of nucleoprotein with traces of ether-extractable substances and salts. A small amount of carbohydrate was present, but larger amounts were found to be liberated apparently from the virus particles during the course of washing. A variety of indirect evidence suggests that this easily lost carbohydrate is in the form of immunologically active polysaccharide of the type rendered familiar by studies on bacterial antigens.

Most viruses which have been tested for their susceptibility to the action of purified trypsin are relatively unaffected, viz., vaccinia, Rous and Fujinami fowl tumours (Pirie, 1935), swine influenza and equine encephalomyelitis (Merrill, 1936), but pseudo-rabies virus is destroyed (Merrill, 1936).

None of the viruses has been shown to multiply in the absence of living susceptible cells, and several of the larger ones are known to produce definite intracellular micro-colonies; the cytoplasmic inclusions of vaccinia, ectromelia, canary-pox and fowl-pox are of this nature. It is logical to believe that all the

viruses have a similar intracellular habitat. Their inability to multiply outside the living cell may well be due to the fact that they are dependent on utilizing the metabolic activities of the parasitized cell to provide them with material for their growth and multiplication. This necessity to enter the susceptible cell probably plays an important part in determining the result of contact with immune serum on the virus.

2. Bacteriophages.

Ultrafiltration methods have also been applied to a series of bacteriophages, and the results are given in Table 2. This table at once disposes of two fallacies still widely current about these agents. The first is that there is a single bacteriolytic agent, the bacteriophage: on the contrary, there are enormous numbers of bacteriophage types readily distinguishable by suitable functional tests, as well as by the size differences shown in this table. The second is that phage is an unorganized substance. The fact that the particles of the commonest bacteriophages are more than $0{\cdot}05\mu$ in diameter and can be seen and counted with the ultramicroscope (Schlesinger, 1933) or photographed by Barnard's methods with mono-chromatic ultra-violet light (Burnet, 1933) shows that they must have a relatively complex organization, and are clearly not enzymes or simple proteins.

Table 2.

Sizes of Bacteriophage Particles.

Phage	Diameter of particle (mμ)
Staph. K., D.4, D.12, C.16, W.L.L. (Schlesinger)	65
D.54, S.41, L.	49
C.36, D.13, D.20, D.48	28
C.13	19
S.13	10

Sufficient amounts of bacteriophage particles have been collected by centrifugation or chemical precipitation in the case of two of the large particle bacteriophages to allow preliminary studies of their chemical composition. Schlesinger (1934 and 1936) used the coli phage W.L.L., which, in regard to size, functional activity and antigenic type, is nearly identical with the phage C.16 used in our own work. He finds this is composed mainly of nucleoprotein-like material which gives a strong Feulgen reaction. There are traces of ether-extractable substances, some inorganic material, and a good deal of carbohydrate, which is readily lost by washing. The large particle staphylococcal phage K. also gives a positive Feulgen reaction. Northrop (1936) has recently published a preliminary note on the chemical nature of this latter phage, finding, like Schlesinger, that it is of predominantly protein composition. It is noteworthy that Schlesinger found that the Feulgen reaction was not given by any constituent of bacteria in the absence of phage.

As regards the mode of action of phage, the original conception of d'Herelle still provides the most adequate description. A phage particle is first specifically adsorbed to the susceptible bacterium, enters its substance, and there multiplies until a certain number, perhaps as many as 200 descendant particles, are present. The bacterium up to this stage retains its form, and none of the descendant phage particles is liberated, until it suddenly disintegrates. Krueger and Northrop (1930) disagree with this interpretation, but their view that a partition relationship exists between intracellular and extracellular phage is quite inconsistent with the results obtained in the type of experiment used by Burnet (1929). Further aspects of the interaction between phage and bacterium which are relevant to immunological studies are more conveniently discussed later.

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Chapter II.

MODERN VIEWS ON THE ANTIGEN-ANTIBODY REACTION.

The particular problems involved in virus-antibody reactions must be only special examples of principles applicable to all antigen-antibody reactions. As a basis for the discussion of these principles we shall adopt the presentation given by Marrack in "The Chemistry of Antigen-antibody Reactions" (1934). This is largely derivative from the work of Landsteiner and colleagues on the one hand, and of Heidelberger and other workers on the bacterial polysaccharides on the other, but makes an important and fertile suggestion in the "lattice" hypothesis. The following summary is essentially an abstract of the relevant portions of Marrack's monograph, from which experimental details and references to the literature may be obtained. Wherever ideas other than those expressed by Marrack are introduced, appropriate references are given.

Circulating antibody consists of serum globulin molecules, the surface of which carries one or more specifically modified "patches." The arrangement of atoms and the associated electrical fields on such a patch are adapted to unite specifically with one or other "determinant group" of the antigen which stimulated the production of the antibody in question.

Antigens are large molecules or aggregates of molecules, usually, but not necessarily, composed partly or wholly of protein, and presenting on their free surface a number of "determinant groups." These may be of very varied chemical nature, and are not necessarily of highly complex structure. It has been shown, for instance, that suitable conjugation of *d*-tartaric acid to protein provides determinant groups of different immunological specificity from those obtained by conjugation of *l*-tartaric acid to similar protein. The high immunological reactivity of some bacterial polysaccharides has been ascribed to their being composed of a long series of aldobionic acid units, each of which can serve as a determinant group.

The processes by which inoculation of antigen results in the appearance of circulating antibody are unknown, largely because practically nothing is known of the way in which the normal serum globulin is synthesized in the body. A useful, but entirely conventional simile is to regard each determinant group as impressing like a die on wax a complementary and permanent image of itself on some of the globulin molecules being produced during the period of immunization. It is important to grasp the fact that not all the antibody molecules produced in response to inoculation with a pure antigen carry active groups corresponding to all the different determinant groups on the antigen molecule. Suppose an antigen molecule has the following ten determinant groups: a.a.a.a., b.b.b., c., d.d., and that

on the average each modified globulin molecule has six active patches. Such antibody molecules as A.A.A.A.B.C. and A.A.B.B.D.D., where A.B., etc., are the modified patches corresponding to a.b., etc., determinant groups, will appear and can readily be separated by appropriate absorption experiments. Probably all populations of antibody molecules are heterogeneous, irrespective of whether the antigen was a single molecular species or not.

Antigen-antibody reactions *in vitro* result from union between complementary determinant groups on the antigen and active patches on the antibody molecule. It is immaterial whether this union is called chemical or adsorptive. When an antiserum reacts with a simple haptene solution, i.e. one in which each active molecule carries *only one* determinant group, the reaction is very incomplete, and *completely reversible*. Under the usual conditions of *in vitro* immunological reactions, relatively concentrated reagents are used, each antigen molecule or particle carries numerous determinant groups, and each antibody molecule has many specific patches. Provided the relative concentrations of the two reagents are not too disparate, a lattice structure forms in which each antigen molecule is united to several antibody molecules. Even if each single bond of union is weak and the corresponding reaction reversible, the formation of a complex lattice will obviously give a much less easily reversible reaction. As the lattice structures increase in size the contained antibody globulin becomes less hydrophilic, and takes on the character of a denatured protein. Provided the salt and H. ion concentrations are suitable, precipitation of the complex eventually occurs.

With simple haptenes, no precipitation occurs, since lattice formation is impossible, each "antigen" molecule being capable of uniting with only one active patch. The conditions become somewhat similar when we deal with extremely dilute antigen, in which the antigen molecules or particles are so far apart that within ordinary periods of time there is only an infinitesimal chance of their colliding with each other. If antibody is in excess, union will occur and be followed by dissociation, an equilibrium condition developing, but there will be no lattice formation to favour union as against dissociation, and there will be less opportunity for globulin denaturation to occur. Such systems can be studied only when we have an extremely sensitive method of detecting traces of antigen. In work with viruses and bacteriophages we can detect a single antigenic particle, and in immunological studies habitually work with concentrations, which in terms of the actual mass of virus particles are almost infinitesimal. As an example, the usual concentration used in our bacteriophage inactivation experiments was approximately 1 in 4×10^{12} by weight. In such systems we can eliminate the possibility of lattice formation playing any significant part. Whether under such conditions the union can become stabilized with time is an important question. Theoretically, if two determinant groups on the antigen particle unite, each with an active patch on one and the same globulin molecule, the chances of dissociation

will be greatly reduced. If this is to occur to any significant extent, there must be a relatively high probability that a second "chance" contact between the virus surface and portion of the antibody molecule will bring complementary active areas together so as to allow secondary union. This condition is most likely to be fulfilled if (a) the antigen particle carries numerous similar determinant groups rather than a wide range of different determinants, each in relatively small numbers, and (b) the antibody molecule also possesses numerous specifically modified patches corresponding to the dominant antigenic determinant of the virus particle.

Although it has not yet been proved that any virus antigens contain complex polysaccharides of the type familiar from immunological studies on bacteria, it is probable that this is the case with some at least. Vaccinia virus is in so many respects like a minute bacterium that it is reasonable to expect that further work will confirm the current impression that polysaccharide components form part of its antigenic structure. Attention will, therefore, be drawn to certain points in regard to bacterial polysaccharides which appear to be relevant to virus problems. The fact that from almost all pathogenic bacteria complex polysaccharide gums of high immunological reactivity can be isolated is well known. Recent work is making it clear that the purified polysaccharide haptenes represent only fragments of the antigenic surface structures of the living bacterium. Several more complex entities have been described which show a closer approach to the natural antigen, such as the acetyl polysaccharide of pneumococcus I, and the polysaccharide- lipoid complexes obtained from various Salmonellas by Boivin and his collaborators (1933). In general it is found that if A., B., C. and D. are successive stages in purification from the living surface A. to the pure polysaccharide haptene D., all these are precipitated by anti A. serum. Absorption of anti A. serum with D. will remove all activity against D., but not against A.B. or C., absorption with C. will remove all activity against C. and D., not against A. and B., and so on. With each step from the natural living surface there has been a loss, large or small, of certain of the determinant groups which give rise to the whole population of antibody in the "natural" immune serum. Considerations of this sort tend to make rather unreal any discussion as to which of a series of bacterial fractions is of predominant immunological importance, whether agglutinating antibody is the same as protective antibody, and so on.

An aspect of immunological reactions which has recently attracted attention is the difference between avid and non-avid antitoxic sera. With the majority of diphtheria antitoxic sera the relation between the L.f. titre (Ramon flocculation test) and protective power (L. + or L.r.) in guinea-pigs is a constant one. Further, the law of neutralization in constant proportions holds over a wide range of concentration. Glenny and Barr (1932) found that certain batches of antitoxin (a) showed a higher antitoxic value by flocculation test than by guinea-pig inoculation

tests, (b) produced, when mixed with certain proportions of toxin, peculiar "ring" type reactions on intracutaneous inoculation, and (c) gave mixtures with toxin, which, inoculated intravenously in *large* amount into rabbits, were non-toxic, but in small doses were rapidly lethal. Such antitoxins were regarded as non-avid, i.e. combining less firmly with toxin than normal "avid" antitoxin. Recently, Morgan and Graydon (1936) have described differences between batches of α-staphylococcal toxin, which appear to be essentially differences in avidity for antitoxin, although the authors themselves consider that the differences may be due to the presence of two distinct toxins. Their avid (α^1) toxin was neutralized by a certain unmodified antiserum and also to the expected degree by a preparation of purified antitoxin globulins from this serum. Non-avid (α^2) toxins, on the other hand, were neutralized by natural serum, but to a very much smaller extent by the purified antitoxin globulins. If this interpretation is correct, we must adopt the view that avidity is entirely a relative term, high avidity of antigen may compensate for low avidity of antibody, and vice versa.

Though the concept of avidity has not received much theoretical study, it is of obvious importance, and it will be found necessary later to invoke it to co-ordinate certain aspects of the immunological reactions of viruses and bacteriophages. The physico-chemical basis of diminished avidity will probably vary in different cases, and too little is known as to the actual nature of antigen-antibody union to make any detailed suggestions about factors which would render it more or less readily dissociable. In very general terms, slight deviations on either side from the normal "complementary patterns" of atomic arrangement may be expected to diminish avidity. Variations in the configuration of either molecule, according to whether it is in free solution or forms part of some solid-liquid or liquid-air interface, may have an important influence on the ease with which union occurs. Finally, where antigenic determinants form part of a living surface, their reactivity with antibody may be modified by localized changes in permeability or degree of hydration.

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Chapter III.

IMMUNOLOGICAL REACTIONS OF BACTERIOPHAGES.

Introduction.

The fact that a bacteriophage could function as a specific antigen was first established by Bordet and Cuica (1921). They showed that, following repeated injections of lysed culture, the serum of a rabbit developed a power to inactivate the homologous phage. The inactivation could be demonstrated by either of the methods ordinarily used to show phage action, i.e. in broth culture or on agar. Bordet and Cuica also found that this inactivating power was not present in anti-bacterial sera produced by immunization with the susceptible bacterial culture.

Since 1920 there has been a copious output of papers on the phage-antiphage reaction. Many of the earlier ones are difficult to evaluate, owing to uncertainty as to whether "pure" phages were being used, and most of the data to be used in this review will be drawn from recent work. Nearly all the work to be quoted in any detail has been carried out either at the National Institute for Medical Research or in this laboratory with the series of phages originally isolated by Burnet and McKie (1930). There are obvious advantages to be gained from centreing the discussion on one well-studied group of bacteriophages rather than from accumulating the results obtained with different phages, many of them very incompletely characterized. Practically all the points to be dealt with have, however, been studied in other laboratories, and brief reference will be made to these investigations in the appropriate sections.

1. The Specificity of Bacteriophage Inactivation by Immune Sera.

It is unnecessary to elaborate the statement that phage-neutralizing antibodies are unrelated to any constituent of normal bacteria. Immune sera always contain bacterial antibodies, but these can be removed by appropriate absorptions with the corresponding bacteria without any diminution in antiphage activity. All workers are agreed on this point.

The problem of the serological relationships amongst different bacteriophages is a more complicated one. Despite certain statements in the early literature to the contrary, a bacteriophage retains its serological specificity irrespective of the bacterial species at whose expense it has multiplied. Its serological nature is one of the most definite intrinsic characteristics of a bacteriophage, and comparative serological work provides the best basis for a classification of any large group of bacteriophages. Burnet (1933d) concluded that while one hardly ever found two bacteriophages which were completely identical serologically, yet the whole

group of coli-dysentery phages could be divided into a small number of serological types. Each type included all those phages which showed any degree of cross neutralization. No overlapping between types was found amongst 50 phages tested. The groups of phages delimited in this way were always composed of functionally similar phages of the same particle size.

The differences within a serological group were of the same type as are found with related but not identical bacteria. The homologous phage is practically without exception more readily neutralized by immune serum than any heterologous phage. When antibody absorption experiments are made, using phage-coated bacteria as the absorbing material, the homologous phage reduces the titre of the serum equally for itself and for heterologous phage. A heterologous phage greatly reduces the titre for itself, but leaves the activity of the serum against the homologous phage almost unaltered (Burnet, 1933a).

2. DIFFERENCES IN ANTIGENICITY AMONGST BACTERIOPHAGES.

On the whole, bacteriophages are excellent antigens, and with many it is easy to obtain antisera from immunized rabbits which will produce considerable inactivation when diluted 1 in 20,000. With some, however, it is almost impossible to obtain an active immune serum, and intermediate cases exist. Absence of inactivating power in the serum after a suitable course of inoculations may mean either that the bacteriophage used is non-antigenic, or that antibodies are produced but are incapable of inactivating the phage. There is no positive evidence available to decide between these alternatives. If antibodies were produced in normal amount, but had no action on the homologous phage, one would expect to find some instances in which antiserum had a greater effect on a heterologous phage than on its specific one. We have never encountered such a relationship in a fairly wide experience of serological work with many different phages. In the circumstances, it is probable that poorly inactivated phages such as D.20 of Burnet and McKie's (1930) series are such because they lack antigenic power rather than because they are resistant to inactivation. In support of this view, we can provide one small piece of relevant positive evidence from the following experiment:

Two rabbits were inoculated intravenously with a mixture of equal volumes of three phages: C.16 (particle size 60 mμ), D.20 (25 mμ), and C.13 (17 mμ), and tests of blood taken from the other ear were made at intervals. All phages were still detectable in the blood at six hours, but only the smallest particle phage (C.13) at 24 hours. Another injection was made a week later, when both rabbits showed antibody titres of about: C.16, 100; D.20, nil; and C.13, 20 units. Phage C.16 was not detected, even in the immediate bleedings, C.13 was not found after two hours, and D.20 persisted in one from two to four hours, and in another from four to six hours.

In addition to demonstrating the rapidity of the primary antibody response to a good antigen, this experiment shows that D.20 not only provokes no detectable neutralizing antibody, but is also not so readily removed from the circulation of the immunized animal.

Limited experiments gave some indication that poorly antigenic phages are also less reactive in other respects. D.20, for instance, although an active large plaque phage, is not readily adsorbed by killed susceptible bacteria, and is quite unaffected by bacterial extracts with high phage-inhibiting activity (P.I.A.) against other phages lysing the same bacterial strains.

3. Antigenicity of Inactivated Phage.

As in so many other aspects of bacteriophage work, there are discrepancies in the literature in regard to the antigenicity of phages inactivated by physical or chemical means. This confusion is largely due to the failure to realize that bacteriophages comprise a highly heterogeneous assemblage of agents differing greatly in their size, resistance to physical agents, and, in fact, in regard to almost every demonstrable characteristic.

Heat-killed phage, according to Schultz, Quigley and Bullock (1929), is non-antigenic. They used 80°C. for one hour, testing a staphylococcal and a dysentery phage. Arnold and Weiss (1924) found that an uncharacterized phage was antigenic after 1½ hours' heating at 85°C., or 45 minutes at 100°C. Muckenfuss (1928) found that a phage (also unnamed) was antigenic when inactivated by moderate heating, but not after boiling or autoclaving.

All are agreed that formalin-inactivated phage is antigenic (Schultz, Quigley and Bullock, 1929, and Muckenfuss, 1928). In some unpublished experiments on the action of formalin on phage C. 16, Burnet found that the rate of inactivation was greater at an acid reaction (pH 6) than at an alkaline one (pH 8·3), reversing the conditions found with toxins. It was much more rapid at 37°C. than at lower temperatures. Preparations inactivated at different reactions and different temperatures with 0·1 per cent. formaldehyde were inoculated into guinea-pigs. All were actively antigenic, and no significant advantage could be shown for any of the methods of inactivation used.

In one experiment, also unpublished, Burnet and McKie obtained a high titre antiphage serum by immunization with a typical *Staph. aureus* phage inactivated by 0·5 per cent. phenol at 37°C.

Schultz, Quigley and Bullock (1929) found that staphylococcus phage inactivated by the photodynamic action of methylene blue was antigenically inert. This experiment was done before the photodynamic nature of the inactivation was understood, and it is probable that the action was allowed to proceed far longer than was necessary to inactivate. Lush (unpublished) has made a fairly extensive study of the antigenicity of phage inactivated by methylene blue 1 :100,000 when exposed in shallow layers to a 200-watt lamp at a distance of 20 cm. Great variations in the time of exposure necessary to inactivate different phages completely were found, from less than 10 seconds to 15 minutes. The immunizations were carried out with material exposed for twice the time necessary to inactivate. Four

phages, normally active antigens, including a staphylococcal phage, Au. 2, and large, medium and small particle dysentery phages (C.16, S.18, and D.44), all gave rise to highly active antisera of titre above 2,000. Two phages less readily antigenic, L., which usually produces rather weak sera, and D.20, which produces only traces of antibody, were also tested for antigenicity after inactivation by methylene blue. No change from the normal active phage was manifested, L. producing a serum of titre 500, and D.20 one of 20 units only. The serum produced by photodynamically inactivated phage C.16 was used in some detailed studies of phage inactivation, and appeared to be entirely similar to sera produced by immunization with active phage.

Production of specific antibody is therefore in no sense an exclusive property of active phage. Inactivation by formalin or photodynamic inactivation with methylene blue does not modify the antigenicity of phage to any significant degree.

4. General Character of the Reaction Between a Typical Large Particle Phage and a High Titre Homologous Antiserum.

It will probably be best to give first a general survey of the phage-antiphage reaction in terms of the phage C.16 and its corresponding antiserum. This phage belongs to the commonest serological group of coli-dysentery phages, its size is known, and much of the recent immunological work has been done either with this phage or the closely related W.L.L. phage of Schlesinger (1934).

If 1 c.c. of a high titre (10^9–10^{10}) filtrate of this phage is inoculated intravenously into a rabbit, considerable amounts of antibody can be detected in the serum after a week, and if two or three subsequent weekly injections are made, a high titre serum is obtained. A final serum dilution of 1 :10,000 will usually reduce the plaque count of dilute phage to 10 or 20 per cent. after an hour at 45°C. The activity of the antiserum can be demonstrated in three ways, (A) by the neutralization technique, (B) by the appearance of macroscopic phage agglutination, and (C) by the agglutination of phage-coated bacteria.

(A) *The phage neutralization reaction.*

Although the term "neutralization" is in common usage, both for toxin-antitoxin reaction and for the interactions between viruses or bacteriophages and their corresponding antisera, it is a misleading one. There is no real analogy with the chemical reaction between acids and alkalies, from which the term has been borrowed, and it is advisable to replace it by the noncommittal "inactivation".

Although broth culture methods may be used, plaque counting methods are more convenient and accurate, and in our own work have been used exclusively. Phage C.16 is a very satisfactory one for such titrations, producing very distinct, easily counted plaques about 0·5–1 mm. in diameter, 200–300 of which can be readily counted on a quadrant of agar.

The following is a brief account of the technique used in inactivation experiments with phages. The stock phage has been titrated previously, and such a dilution is prepared that when it is mixed with an equal volume of broth and plated in the usual volume of 0·02 c.c., 100–150 plaques will develop. This is the "standard dilution." For an ordinary serum titration, or for the estimation of the activity of any other phage-inactivating agent, a series of tubes is prepared, containing each one volume of standard dilution and an equal amount of one of a set of dilutions of serum, etc., in broth. The tubes are well shaken and then placed in a water bath at the desired temperature for the time required. At the end of this period they are chilled in ice water, and 0.02 c.c. amounts delivered from a calibrated capillary pipette on to plates previously spread with a few drops of young broth culture of the sensitive strain, for C.16 the original Hiss and Russell strain of Y. dysentery bacilli (Y.S.). The plates contain 1 per cent. of nutrient agar, and are left in the incubator overnight before being used. This ensures that both the initial spreading of culture and the bacteriophage mixture will dry off rapidly. As soon as the inoculated plates have dried, their tops are lowered and they are incubated overnight. The titre of the serum is expressed as the reciprocal of the final dilution of serum capable of reducing the plaque count to 20 per cent. of the control. For more detailed experiments the same technique is used, with the necessary modifications in concentration of reagents, time of action, etc., which may be required. In all experiments it is the aim to obtain counts of 20–100 plaques on the experimental platings.

To analyse the reaction we require information particularly in regard to (i) the degree to which the reaction is reversible by dilution or other means, (ii) the progress of the inactivation with time, and (iii) the quantitative relation between the concentrations of reagents and the number of plaques resulting when the conditions and time of contact are the same. In practice these points cannot be clearly separated, but some experiments supply more information about one than about the other.

(i) *The degree of reversibility.* Practically speaking, the inactivation of phage C.16 by immune serum is irreversible by dilution. If a mixture of relatively concentrated reagents gives 100 plaques, a tenfold dilution of this mixture will give perhaps 20 plaques, a hundredfold dilution, 2. This apparent reactivation is probably due to two factors which come into play when relatively concentrated mixtures of phage and serum are placed on the agar surface of titration plates. The first is the continuing inactivating effect of the antiserum which may proceed until effective contact of all uninactivated phage particles with susceptible bacteria has been accomplished, and the second is the increased opportunity for aggregation of antibody-coated phage particles during the short period of drying. With initially more dilute reagents no reactivating effect with dilution can be demonstrated. In the absence of significant reversibility, it becomes legitimate

to titrate serum-phage mixtures by plating dilutions made after the reaction has been in progress for the desired time. It is unlikely that the reaction is absolutely irreversible, and evidence will be given in a later section that by certain methods some dissociation can occur, but this does not invalidate the conclusion that for all practical purposes the reaction may be regarded as an irreversible one.

(ii) *The influence of phage concentration.* Working mainly with another phage (C.36), but for some experiments also using C.16, Andrewes and Elford (1933) showed that over a wide range of serum concentrations the proportionate reduction in the number of active particles (plaques) was independent of the phage concentration.

We can confirm these observations for several other phages with which we have worked, and Table 3 provides a good example of the accuracy with which the rule holds for the inactivation of phage L. by its homologous antiserum.

TABLE 3.

To Show the Percentage Law.

Antiserum Dilutions	Phage Dilutions			
	1: 10^2	1: 10^3	1: 10^4	1: 10^5
Undiluted	289	23·3	2·0	
1: 3	675	80	10	
1: 10		142	16·7	1·7
1: 30		243	26	2·0
1: 100		>1,000	145	11·3
1: 300			296	33
1: 1,000			356	36·3
Control			570	51

Phage L. with homologous antiserum 4 hours at 37°C.
Plaque counts given are averages of three platings.

In practice, deviations from this relationship may be observed with very low serum concentrations, or with mixtures in which both reagents are in high concentration, but over the usual range of concentrations studied, this "percentage law" holds, i.e. if a certain concentration of serum reduces the count from a "standard dilution" of phage (approximately 1:1,000,000 of stock filtrate) to 10 per cent., it will also reduce the count from undiluted phage to 10 per cent. As Andrewes and Elford point out in their discussion, the "percentage law" is merely an indication that under the usual conditions of experiment, each phage particle may be regarded as an isolated individual surrounded by an unlimited volume of antibody solution. This point of view will be discussed in detail later. The "percentage law" may break down at extremes of concentration, but over the usual range it may be accepted as a working rule which greatly simplifies the design and interpretation of experiments on other quantitative aspects of the reaction.

(iii) *Influence of serum concentration and progress of the reaction with time.* These two aspects are most conveniently considered together, since the essential influence of change in immune serum concentration is to modify the *rate* at which inactivation proceeds. Figure 1 shows time survivor curves for various dilutions of antiserum at 37°C., the "percentage law" being assumed to hold throughout.

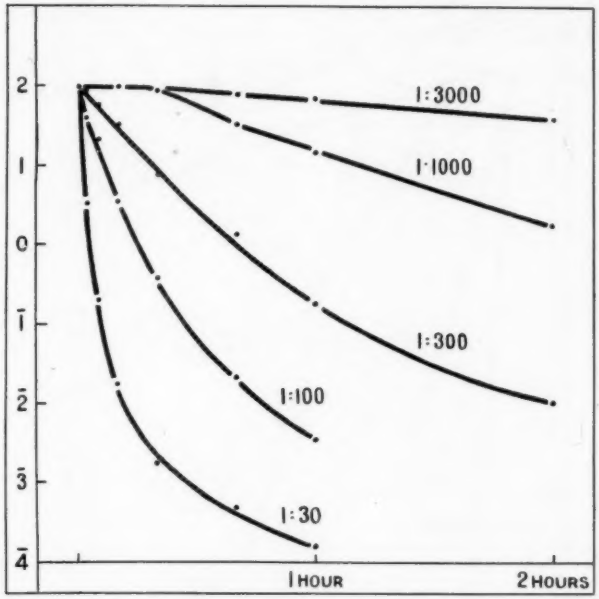

Fig. 1. Time-survivor curves for phage C.16 at 37°C. with the dilutions of antiserum shown. The logarithm of percentage survivors is plotted against time in hours after making the mixtures.

The data for this figure were obtained from experiments in which a constant serum concentration was mixed with various phage dilutions, so arranged that at each time interval at least one mixture should be available which, on plating direct, would give a countable number of plaques. This is an orthodox method of carrying out such experiments, but it must be remembered that in all cases there is a continuing action of the serum on the surface of the plate, and that this will be greater with higher serum concentrations.

This effect can be eliminated by appropriate dilution before plating, and in Figure 2 we give the results obtained with the same serum and phage at 37° C. when all mixtures with serum concentration above 1:1,000 were diluted in cold broth immediately before plating to reduce the serum concentration to 1:1,000

or less. For reasons to be discussed later, this experiment was concerned only with the earlier stages of the reaction, during which the count fell from 100 per cent. to about 1 per cent. It will be seen that there is a much better approach to a logarithmic course when the experiment is conducted in this fashion, and it is also

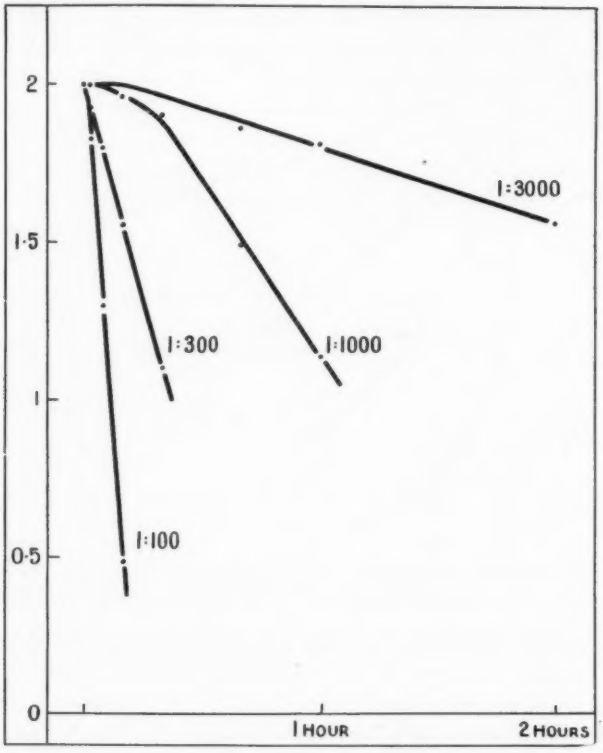

Fig. 2. Time-survivor curves for phage C.16 at 37°C. with antiserum dilutions 1: 100 to 1: 3,000. In this experiment all mixtures were diluted to reduce the serum concentration below 1: 1,000 before being plated, thus eliminating the effect of continuing serum action on the agar surface.

clear that with very dilute serum there is a distinct lag period before the reaction takes on its logarithmic phase. If we are to compare the activity of different serum concentrations, the most suitable measure is the rate of the reaction during its logarithmic phase, as determined from experimental data of the type used in Figure 2.

Such a method requires a fairly elaborate experiment, and a good deal of useful information can be obtained from experiments along orthodox lines. It will be seen from Figure 1 that the shape of the curves varies according to the concen-

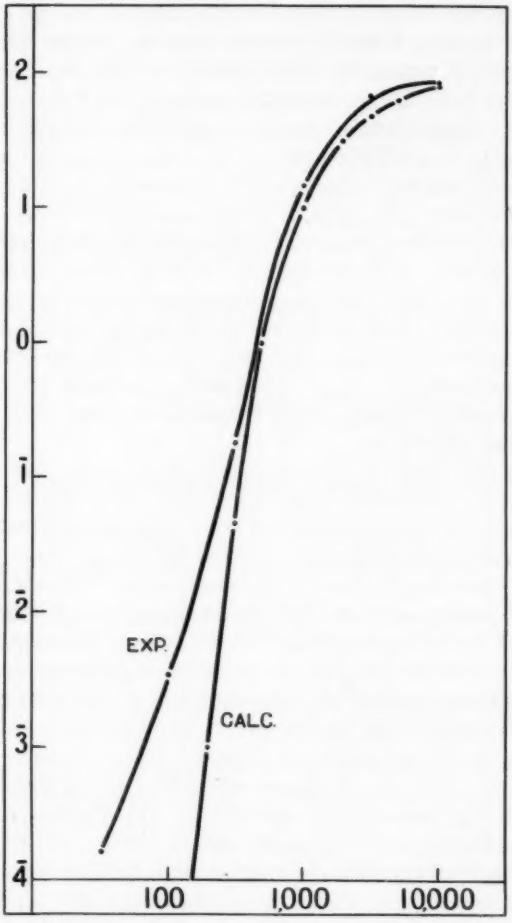

Fig. 3. Serum dilution-survivor curve for phage C.16, 1 hour at 37°C. Calculated curve is plotted on the assumption that rate of inactivation follows a logarithmic course and is proportional to the concentration of serum.

tration—with strong serum there is a precipitous initial fall, followed by a steady slowing in rate, while at limiting dilutions there is an initial lag before an approximately steady rate is reached. It follows that the shape of serum concentration-

survivor curves will vary according to the time and temperature conditions chosen. One hour's contact at 37°C. is a usual arbitrary combination, suitable amounts of phage being added to tubes containing a series of serum dilutions and the mixtures being plated at the end of the period without dilution. Figure 3 represents the serum concentration-survivor curve of phage C.16 under these conditions. On the same Figure is a curve calculated on the basis that the phage population is homogeneous, and that inactivation proceeds logarithmically at a rate proportional to the concentration of the serum. There are two systematic deviations of the experimental from the calculated curve. With the more dilute serum the reduction in plaque count is less than would be expected, but the curves run almost parallel. This is mainly due to the existence of a lag period with dilute sera before the approximately logarithmic course of the reaction becomes evident. As the serum concentration is increased and the proportion of survivors becomes much smaller, there is a much greater discrepancy between the curves. There are many more survivors than would be expected from the calculation. Since under the conditions of experiment other factors would tend to increase the apparent inactivation, this deviation can be safely regarded as due mainly to heterogeneity amongst the phage population, a manifestation of the abnormally high resistance to inactivation of a proportion of the phage particles.

(B) *Agglutination of Phage Particles.*

It was shown independently by Burnet (1933c) and Schlesinger (1933) that large particle high titre phages could be agglutinated by homologous immune serum. In order to observe the phenomenon, it is essential to use a high titre phage containing at least 10^{10} particles per c.c. When such a phage filtrate is mixed with about one-tenth of its volume of antiphage serum, from which all bacterial antibodies have been absorbed, and held at 45° C. for some hours, first an opalescence and then a very finely granular turbidity appears in the mixture. The bulk and coarseness of the final precipitate are directly proportional to the concentration of phage present.

The evidence that this precipitation is a true agglutination of phage particles, and not an associated precipitin reaction of some antigen or haptene in solution, is as follows: It is obtainable with phage particles washed by Elford's methods over an impermeable membrane and with phage grown on antigenically dissimilar strains of bacteria. Conversely, it is not obtained with ultrafiltrates of phage C.16, nor with antigenically dissimilar phages grown on the strain Y.S. By direct observation of phage-immune serum mixtures under the ultramicroscope, Schlesinger could follow the process of aggregation of the diffracting granules which he had previously shown to represent the actual phage particles. The aggregates from C.16-immune serum mixtures obtained in Burnet's experiments were photographed by Barnard, using his ultraviolet

technique, and shown to consist of aggregations of particles. The individual particles were incompletely resolved, but their appearance was compatible with that of bodies of the size 0.05–0.075 μ which had been ascribed to phage C.16 by Elford.

The phages used by Schlesinger and Burnet were of the same serological type, and up to the present no report of agglutination of other types of phage has appeared in the literature. The common small plaque staphylococcal phages are of the same particle size as C.16, and we have found in this laboratory that high titre phage of this type can also be agglutinated by immune serum. None of the smaller particle phages which we have examined showed any agglutination. This is to be expected from the simple considerations in regard to number and size which have been recently discussed by Merrill (1936). There is insufficient mass of antigen to provide visible aggregates.

(C) *Agglutination of phage-coated bacteria.*

Most active bacteriophages are readily adsorbed by killed bacteria of a susceptible strain. According to Schlesinger (1932) up to 140 phage particles may be attached to a single bacterium. Bacteria coated with phage in this way show considerably altered properties. Confining our attention to phage C.16, if small numbers of formalin-killed and washed bacteria (Y.S.) are added to strong phage, left for an hour at room temperature and then deposited by centrifugation, the deposit will be found to be much more cohesive than a similar sediment of normal bacteria. When emulsified in saline, the opacity is a good deal less than would have been given by the original amount of bacteria added to the phage, and the suspension is distinctly unstable, depositing almost completely on standing overnight. The immunological character of the suspension is also altered. Agglutinability by homologous antibacterial serum is greatly reduced, and an entirely new susceptibility to agglutination by antibacteriophage serum has appeared. The effect is specific, being shown with the homologous antiphage serum absorbed free of bacterial antibodies and not by antiphage sera of different type. The agglutination is granular rather than floccular in type.

By this phage-coating technique it is possible to concentrate on about 0.1 c.c. of packed bacteria 99 per cent. of the phage present in 100 c.c.. of high titre filtrate. This renders it practicable to perform absorption experiments, and to show directly that the antibody bound by the phage-coated bacteria, and therefore presumably responsible for their agglutination, is identical with the phage inactivating antibody. Cross absorption of sera against related but not identical phages can also be carried out, and in the experiment described by Burnet it was shown that the mutual serological relations of two such phages were of the same type as is observed in regard to agglutinin absorption between related bacterial types. Bacteria coated with homologous phage reduced the titre of the serum

equally for each phage, while bacteria coated with the heterologous phage absorbed antibody active against this phage, but left the titre against the homologous phage almost unchanged.

(D) *Specific Soluble Substance from Phage.*

By passage through a gradocol membrane of suitable permeability, a high titre stock filtrate of C.16 can be rendered free of phage particles, but still left with any soluble substances present in the original stock. Such an ultrafiltrate can be shown to contain serologically reactive material of the same specificity as the phage from which is was derived. The technique was similar to the method used by Landsteiner and van der Scheer (1928) to show the blocking effect of simple haptenes on the reaction between artificial proteins and their corresponding antisera. Two sets of serum dilutions were made, one in broth, one in ultrafiltrate and standard dilution of phage added after an hour. In the presence of ultrafiltrate, the apparent activity of the serum was considerably reduced. The blocking effect was specific, being shown only against homologous antiserum.

The substance responsible for the effect was relatively heat resistant, not being completely destroyed until exposed to a temperature of 90°C. for 30 minutes. This suggests that the substance is more probably of polysaccharide than of protein nature, and it may be identical with some of the carbohydrate which Schlesinger (1934) found to be easily liberated from phage particles during the process of washing used in his chemical studies.

TABLE 4.

Reactivation of "Neutralized" Phage by S.S.S.

Original phage concentration	Secondary dilution in		Equivalent plaques in absence of serum	Final concentration of serum	Time of plating after secondary dilution			
					Immediately	2 hours	4·3 hours	28 hours
10^{-3}	Broth	10^{-1}	$1{\cdot}3 \times 10^{3}$	1: 25,000	800	165	32	0
		10^{-2}	$1{\cdot}3 \times 10^{4}$	1: 250,000	128	76	40	1
	S.S.S.	10^{-1}	$1{\cdot}3 \times 10^{3}$	1: 25,000	*1,000*	*1,000*	*800*	*800*
		10^{-2}	$1{\cdot}3 \times 10^{4}$	1: 250,000	167	208	205	342
10^{-3}	Broth	10^{-1}	$1{\cdot}3 \times 10^{4}$	1: 25,000	119	15	4·5	0
		10^{-2}	$1{\cdot}3 \times 10^{3}$	1: 250,000	14	8	5	0
	S.S.S.	10^{-1}	$1{\cdot}3 \times 10^{4}$	1: 25,000	135	110	113	128
		10^{-2}	$1{\cdot}3 \times 10^{3}$	1: 250,000	12·5	21	22	42

Plaque counts in italics are estimates only; the other figures are averages of two counts.

From Burnet (1933b).

When very highly diluted serum was used, it was possible to show that actual reactivation of neutralized phage could be effected by addition of phage ultrafiltrate. Since this is the only direct evidence that phages of this type are not "killed" by antiserum, a protocol of the experiment described by Burnet (1933b)

is shown in Table 4. It will be seen that the reactivation progresses slowly with time. Incidentally, the protocol shows very clearly the *absence* of reactivation of this phage by simple dilution.

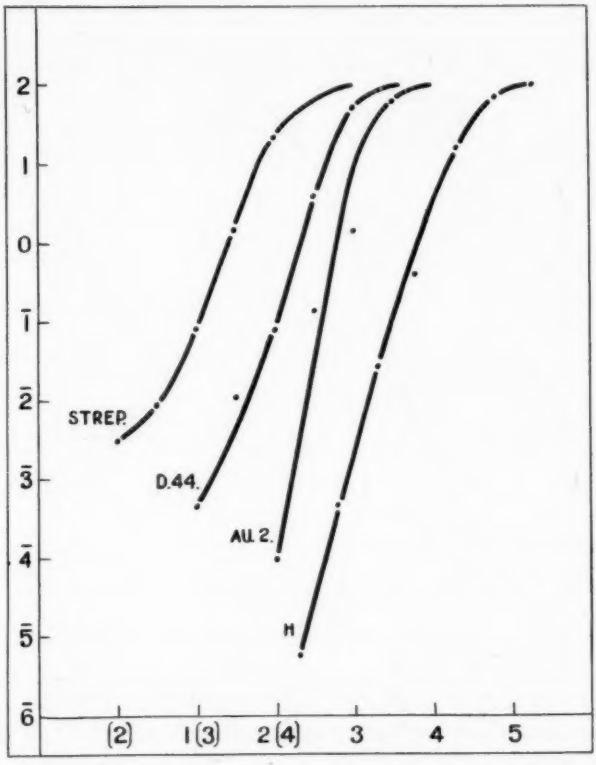

Fig. 4. Composite graph to show general resemblance of serum dilution-survivor curves for distinct types of phage. Strep.: streptococcal phage A. (Evans). D.44: large plaque dysentery phage. Au.2: *Staph. aureus* phage. H.: small plaque dysentery phage. Ordinates: log. percentage survivors. Abscissae: log. serum dilutions; bracketed figures refer to streptococcal phage antiserum only.

5. Other Phage-antiserum Inactivations.

No other phage has been so completely studied immunologically as C.16, but there is no reason to doubt that its reactions are typical of the majority of bacteriophages. In Figure 4 are shown serum concentration-survivor curves of (1) a large particle dysentery phage (H), (2) a small particle dysentery phage

(D.44), (3) a typical phage (Au.2) lysing *Staph. aureus*, and (4) a streptococcal phage. All these phages are "good antigens," and the shape of the curve is very similar in each case.

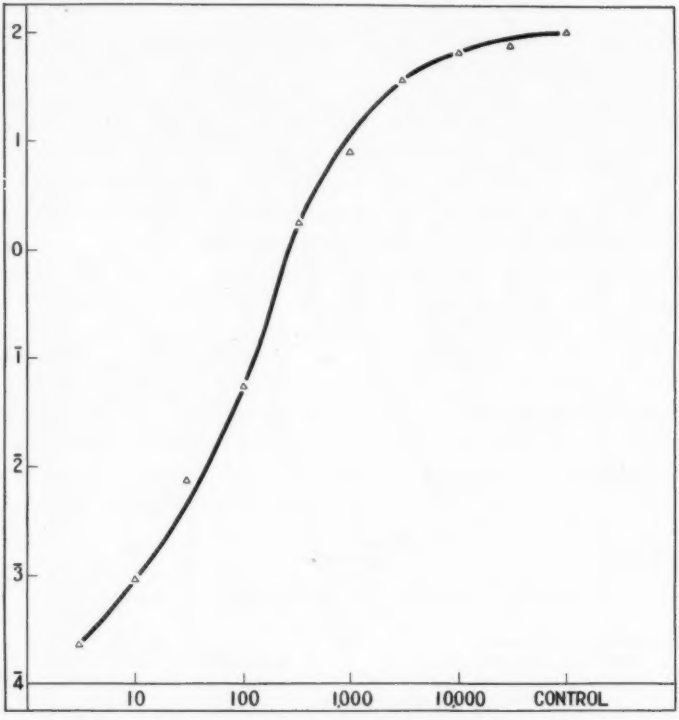

Fig. 5. Serum dilution-survivor curve for smallest particle phage S.13, 1 hour at 37° C.

Even the smallest known phage (S.13, particle size 8–12 mμ) shows essentially similar reactions to C.16. The serum concentration-survivor curve (Figure 5), however, is more definitely sigmoid, indicating probably a greater degree of heterogeneity in the population. The time curve (Figure 6) is typical, and there is no greater evidence of reversibility on dilution than with C.16.

All the phages of the same serological type as C.16 can be similarly agglutinated if of sufficiently high titre, and can also be used to coat sensitive bacteria and to provide specifically agglutinable suspensions. A small-particle phage, C.13, which is serologically distinct, is the only coli-dysentery phage outside the C.16 group which has been shown to endow bacteria to which it has become attached

with a specific agglutinability by antiphage serum. Two groups of staphylococcal phages can also be used to coat staphylococci and induce similar changes in agglutinability (Burnet and Lush, 1935).

The production of a specific soluble substance seems to be a general characteristic of at least the coli-dysentery phages.

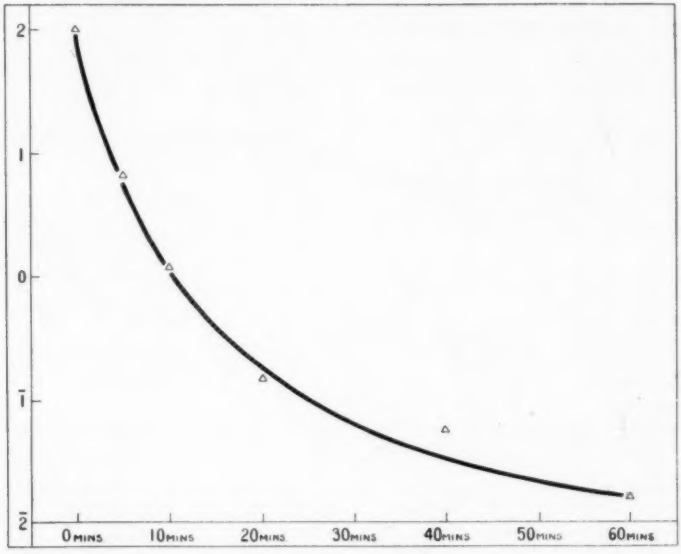

Fig. 6. Time curve of inactivation of phage S.13 by antiserum 1: 30 at 37°C.

There are some less actively antigenic phages whose serum concentration-survivor curves differ sharply from those given in Figure 4. Phage S.18 has been chiefly studied in this laboratory, but phage C.36, used for most of the theoretical work described by Andrewes and Elford (1933) is of the same serological type, and is of generally similar character. The serum dilution-survivor curve shown in Figure 7 is of particular interest from the presence of a distinct "step." This step, which appears to indicate that about half the phage particles are particularly easily inactivated, was apparent in four out of five experiments. The other gave a curve of the same general shape, but lacking any definite step.

6. Evidence for the Existence of "Partly-neutralized" Phage.

Andrewes and Elford (1933), working with phage C.36, obtained evidence from several different types of experiment that the character of phage particles

could be modified by concentrations of immune serum insufficient to inactivate them in the ordinary sense.

In the first place, the "surviving" phage particles in a serum-phage mixture produced plaques of smaller average diameter than normal. This held even when, before plating, the mixture was diluted sufficiently to eliminate any continuing effect of serum during the development of the plaques. The diminished size of the plaques was shown to be due in all probability to a *delay* in the initiation of lysis by partly neutralized phage particles.

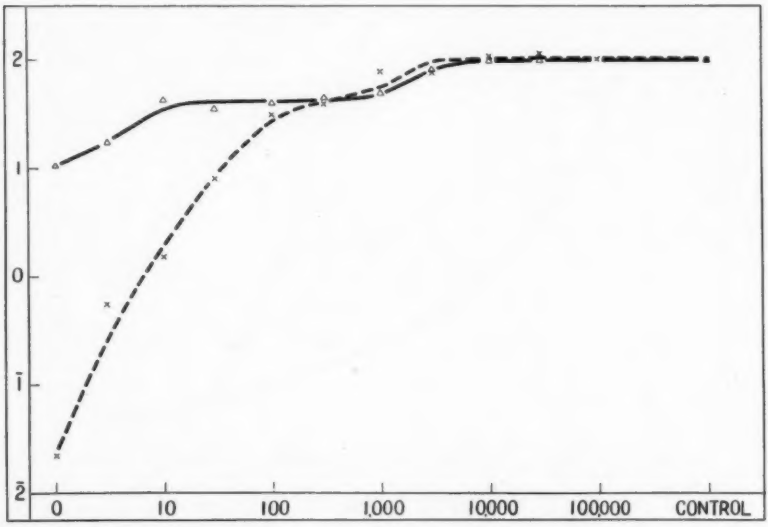

Fig. 7. Serum dilution-survivor curves for phage S.18, 1 hour at 37°C. Two different immune sera are shown, the weaker showing the step referred to in the text.

Secondly, certain phage-serum mixtures containing an excess of viable phage when passed through filters normally permeable to the phage, provide filtrates which are completely free from active phage.

Phage C.16 is a suitable one for the study of variation in plaque size, since the normal phage produces plaques of very uniform size with a sharply peaked frequency distribution curve of diameters. A few experiments in which frequency distribution curves of plaque diameters have been obtained from platings of serum-phage mixtures have revealed some points of interest. To avoid differences in plaque size associated with variations in the medium or conditions of incubation, experimental and control platings were made on sectors of the same plates. Measurements of plaque diameters were made by means of an eyepiece micrometer

and a low power dissecting microscope. A large typical plaque of C.16 has a diameter of 15 units in terms of the micrometer divisions (0·071 mm.) and all plaques were measured to the nearest such unit. The curves are plotted from these results and smoothed by using a moving average over 3 units.

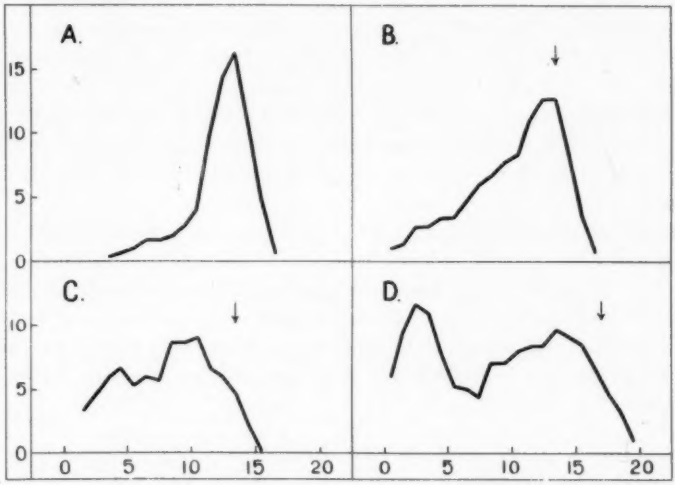

Fig. 8. Frequency distribution of plaque sizes (in arbitrary units) for phage C.16.

A. Normal phage without serum.

B. From a mixture of strong phage + serum 1: 30, diluted 1: 1,000 and plated two minutes after mixing.

C. From a similar mixture plated after 5 minutes.

D. From a mixture of phage with serum 1: 30,000 which had reacted for some weeks in the refrigerator.

In B., C. and D., the arrow marks the peak of curve for normal phage plated as a control on another portion of the same agar plate.

Figure 8 shows the curves: A. for phage C.16 in the absence of serum; B. from a plating of a mixture of strong phage, plus serum 1 :30. After two minutes' contact, the mixture was diluted 1 :1,000 to reduce the plaque count to a convenient number and to eliminate the effect of serum on the plate; C. from a similarly treated mixture which had reacted for five minutes; D. from a mixture of phage and serum 1 :30,000, which had reacted for some weeks in the refrigerator. The arrow shown in curves B., C. and D. represents the position of the peak of the corresponding control curve from normal phage. All these control curves were of the normal form shown in A. In each case two platings of the same mixtures were measured, and both curves shown to be of the same type.

The main interest of these curves is the indication that when the reaction has proceeded far enough to give a reduction to 1 per cent. or less of the original plaque count, a two-humped frequency distribution curve is regularly obtained. The right-hand (large diameter) hump corresponds, to some extent, with the normal curve, but its peak is always displaced to the left of the normal. The left-hand hump represents the "pin point" plaques, and is particularly marked when weak serum has acted for a long period. These reductions in plaque size can only be due to delay in the period required for the initiation of lysis. In all the examples given, the amount of serum added to the plate was too small to have any effect on the development of a plaque once the infection of the initial bacterium affected had occurred. The double-humped nature of the curve strongly suggests that there are two different types or degrees of modification by serum which fall short of complete inactivation.

Recently Burnet and Freeman (1937) have described an experimental method of showing a modified condition in the presence of small concentrations of immune serum, with a phage H. whose immunological behaviour is almost identical with that of C.16. This method is described in a later section, since it depends on the use of phage-inactivating bacterial extracts. Such extracts show many points of resemblance to antisera in their action on phage, and are discussed briefly in the next section.

7. Inactivation of Phage by Bacterial Extracts in Relation to the Phage-antiphage Reaction.

Any theoretical interpretation of the nature of the phage-antiphage reaction will need to take into account that remarkably similar specific inactivating effects on phage can be produced with suitable filtered extracts of susceptible bacteria. Since in a later section great importance is attached to this resemblance between the two reactions, it is advisable to give here a brief account of the activity of the phage inhibiting agent (P.I.A.) of bacterial extracts.

From a consideration of the relations between the antigenic structure of bacteria of the Salmonella group and their susceptibility to phage, Burnet (1927) put forward the hypothesis that phage particles united specifically with the bacterial polysaccharides. Later work with the Flexner dysentery group (Burnet and McKie, 1930) strengthened this hypothesis considerably. It was shown that each of the smooth antigenic types, V, W, X, Y, Z, had a characteristic range of susceptibility to phages, while rough variants obtained from each type were all antigenically similar, and all lysed by the same series of phages. Recently Sertic and Boulgakov (1936), and also Craigie and Brandon (1936) have shown that *B. typhosus* strains, possessing the Vi antigen of Felix and Pitt (1934), differ specifically in their phage reactions from smooth strains lacking this antigen. In each of these cases, and in many other similar examples recorded in the literature,

there is a clear relation between the somatic antigenic structure of the bacterium and susceptibility to phage. Further, since susceptibility to phage lysis is, with very few exceptions, correlated with power of the heat-killed bacteria to adsorb phage, it was logical to assume that phage was adsorbed to the antigenic determinants on the bacterial surface.

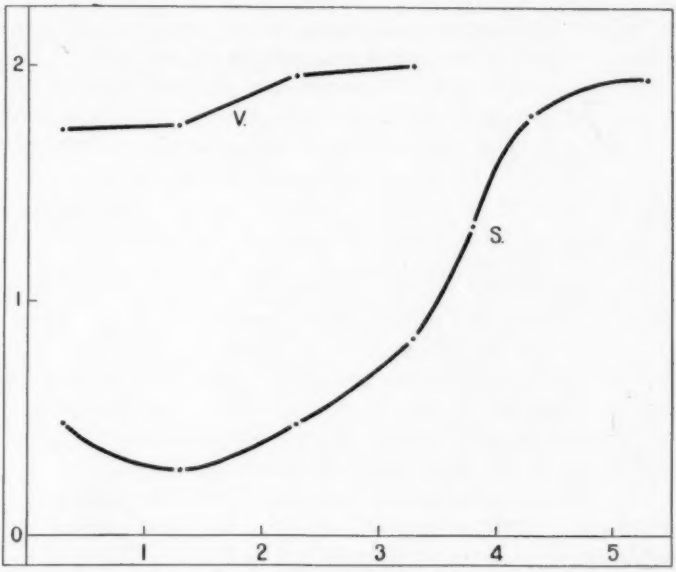

Fig. 9. Effect of P.I.A. on two strains of the same phage H. S. = stock strain, V = variant selected for lack of absorption to susceptible bacterium. Ordinates: log. percentage of survivors. Abscissae: log. dilution of P.I.A.

Levine and Frisch (1933) were the first to show that filtered bacterial extracts could inhibit the activity of phages lysing the corresponding bacterial strain. Burnet (1934a) made a detailed study of the biological aspects of the reaction, and with Gough (1934) showed that the active agent was the bacterial polysaccharide antigen or some more complex molecule containing this. Bruce White (1936) has recently shown that immunologically active polysaccharides of the cholera vibrio play a similar rôle in regard to certain phages lysing that organism. It is characteristic of the reaction that crude filtered extracts are more satisfactory agents than purified materials. If P.I.A. concentration-survivor curves are plotted, the crude extracts show a relatively steep curve, the purified materials show some effect to a high dilution, but the slope of the curve is much flatter.

Confining ourselves to crude extracts, we find that these react with phages in very similar fashion to specific antisera. The reaction is highly specific. If a bacterial variant resistant to the phage in question is developed, the corresponding extract is without inhibitory action on the phage, even though the change from susceptible to resistant strain has involved no detectable antigenic change. The "percentage law" is equally well exemplified, the reaction progresses steadily with time at a rate proportional to the concentration of P.I.A., and does not appear to be reversible. Its speed increases with rising temperature, except for one example, which showed evidence of an optimal temperature around 37°C.

Throughout the work with this phenomenon of inactivation by filtered bacterial extracts, we have been impressed by the evidence of heterogeneity in both components of the reaction. Considering the phage population first, we find that a pure phage obtained by several re-isolations from single plaques may show far from uniform behaviour with a given bacterial extract. Burnet and Freeman (1937) have recently described the reactions of several different stocks of the same pure phage H. Their experiments took the form of determinations of percentage reduction of plaques in the presence of serial dilutions of extract, the technique being identical with that used in immunological work with phage. Figure 9 shows the type of curve obtained with two different stocks. In each case it appears that while a certain proportion of phage particles is inactivated by high dilutions of P.I.A., another proportion is completely insensible. These two levels differ for each stock; in one obtained by a special method designed to select a resistant population, practically all the phage particles remain unaffected.

If a fairly concentrated phage suspension is allowed to react with P.I.A., then diluted to give a "standard dilution" of still active phage, this will be found almost unaffected by a fresh addition of strong P.I.A. There seems to be no alternative to assuming that phage particles differ amongst themselves in some quality which we may term their susceptibility to P.I.A.

There are similar complementary indications of heterogeneity amongst the molecules or submicroscopic aggregations which comprise the specific element of the active bacterial extracts. Certain units are more *avid* than others, in the sense that they will inactivate a phage particle which the latter would not. These differences of avidity are complementary to the differences in susceptibility of phage particles. A highly susceptible phage particle may be inactivated by P.I.A. of high or low avidity, while a less susceptible one will be inactivated only by material of high avidity.

Experimental evidence of these differences of avidity may be found (a) in a comparison of the effect of crude and purified P.I.A. preparations, as mentioned earlier, and (b) by "absorption" experiments. Burnet (1934a) described an attempt to "absorb" out fractions of activity from a P.I.A. preparation active against several different phages, which gave an instructive result. Phages which were readily inactivated did not remove any appreciable amount of activity against

themselves, but the phage C.54, which was affected to a considerably lesser extent, absorbed out this activity almost completely, while leaving the major activity of the extract (against other phages) unaltered. It is evident that only a small proportion of highly avid P.I.A. units are capable of reacting with C.54, and most of these can be removed by absorption with this phage. The more susceptible phages can remove only an average sample of the population, and under the experimental conditions used, the amount removed makes no significant difference to the activity of the preparation. Incidentally, this experiment demonstrates that, as in the immunological reactions, the active agent unites with the phage particle.

Taking into account earlier work on the relation between susceptibility to phage and antigenic structure, the following provisional interpretation of the P.I.A.-phage reaction may be made. The first stage in the lysis of susceptible bacteria requires the mutual specific union of certain elements of complementary molecular configuration on the surfaces of the phage particle and bacterium respectively. The specific element on the bacterial surface is intimately related to the polysaccharide haptene which determines the antigenic character of the bacterium. All the indications are that the haptene, as finally isolated by chemical methods, is not present as such in the living bacterial surface, but forms an essential part of a more complex molecular pattern. It is to certain aspects of this pattern, not necessarily the same for each phage lysing the organism, that specific phage adsorption occurs. Change in susceptibility to phage, e.g. in the resistant variants appearing after lysis, involves change in the surface pattern which may be sufficient to alter the antigenic nature (S. to R. transformation), but in many cases produces no detectable antigenic change in the bacterium. Any process by which the bacterial surface components are brought into solution necessarily destroys to some extent their specific molecular pattern. The bacterial extracts actually used probably contain a highly heterogeneous mixture of fragments of the surface mosaic of varying sizes, and the power of these to unite with and inactivate phage will vary widely, but will always be less than that of the original bacterial surface. Chemical purification methods will in general be designed to separate from the mixture a more or less uniform sample of the smaller polysaccharide molecules, and except for certain phages will reduce the average activity of the material.

The points to be stressed in relation to the phage-antiphage reaction are (1) the kinetics of the reaction are closely similar to those of the immunological reaction, and (2) there is wide variation in the avidity of phage particles of the same pure strain for P.I.A.

8. The Interpretation of Phage Inactivation by Immune Serum.

If we confine ourselves to the purely physico-chemical manifestations of the phage-antiphage reaction, there is no special difficulty in their interpretation. The bacteriophage particles unite with antibody, and under suitable conditions undergo aggregation. When sufficient amounts of phage are used, absorption of antibody

from the serum can be demonstrated. Making allowance for the smaller size of the phage particles, there is no essential difference between the reaction and that of such an organism as the pneumococcus with homologous immune serum. The resemblance becomes even closer with the evidence that soluble specific substance from the phage, quite possibly polysaccharide in nature, can also react with the immune serum.

Aggregation reactions, however, have been very little studied amongst the bacteriophages. Attention has been almost wholly concentrated on the inactivation or "neutralization" of phage by immune serum. Here interpretation becomes much more difficult, for all experiments deal with the combined result of two processes, (1) the union of antibody to the phage particle, and (2) the modification of infectivity produced by this union. The major problem is to elucidate the nature of the second process. The union of antibody to the phage will almost certainly follow the same laws as have been deduced from other immunological studies with more easily manipulated materials. Modification of infectivity in the phage-antiphage reaction, however, cannot be at all closely analogous to the protective effect of serum in mouse inoculation tests with pneumococci, but it may have important analogies with superficially similar serum inactivation effects on animal-pathogenic viruses.

In discussing the phage inactivation reaction, it seems best to deal with certain points individually before attempting any general theoretical account.

(A) *Antibody Bound to the Phage Particle is Responsible for the Inactivation.*

Since in all experiments, phage, serum and susceptible bacteria are present together, it is first necessary to prove that the antiserum acts on the phage directly, and not by rendering the bacteria insusceptible to phage action. That the inactivating effect is entirely a manifestation of union of phage and antibody is immediately evident from the two sets of experimental data showing (a) that the inactivating effect is a function of the time of interaction between phage and serum in the absence of bacteria, and (b) that dilution of a mixture of phage and serum after interaction does not result in any significant reactivation of phage. Further, the fact that by using large amounts of phage (in the form of phage-coated bacteria) the inactivating antibody can be absorbed out of an immune serum, adds direct evidence of union between phage and antibody. In all the subsequent discussion it will be taken as certain that the inactivation is due to the presence of bound antibody on the surface of the phage particle.

(B) *More Than One Antibody Molecule Must be United to Each Phage Particle to Render it Inactive.*

Andrewes and Elford (1933) have shown that with certain phages at least a well-characterized state of "partial neutralization" exists. Its chief feature is a delay in the initiation of lysis most readily demonstrable in plate titrations by a

reduction in the average diameter of the plaques, but also demonstrable by more direct methods. The variations in plaque size of C.16 under the influence of serum were described in section 6, and characteristic two-humped frequency curves of plaque diameters are shown in Figure 8. The interpretation of this type of curve is not yet clear, but it seems that two distinct degrees or types of partial inactivation must be involved. A second feature of partially neutralized phage is its inability to pass certain membrane filters freely permeable to normal phage. Another effect of small amounts of serum on phage has recently been observed by Burnet and Freeman (1937) in the course of work on the phage inhibiting agent (P.I.A.) of bacterial extracts. Phage C.16 is little influenced by P.I.A., and the work was done with a phage of the same serological type (H), which is readily inactivated by dilute P.I.A. A very small amount of serum, enough to reduce the plaque count to 5 or 10 per cent. of the original, is added to phage H, and after an hour the mixture is diluted beyond the range of activity of the serum. The activity of P.I.A. is now tested, both on unmodified phage and on serum-treated phage of equivalent remaining activity. It is found regularly that the serum-treated phage is almost completely insusceptible to inactivation by P.I.A. Suitable quantitative experiments show that this is not a selection effect, but a true modification of individual phage particles by the immune serum. Recent experiments by Miss Freeman have shown that precisely similar phenomena can be demonstrated with a staphylococcal phage and the appropriate antiserum and P.I.A. This phenomenon is interesting from a number of points of view, but in the present connection mainly as indicating that one or possibly more antibody molecules may unite with phage particles and modify their properties without destroying their chief characteristic activity.

It should be noted, however, that all these characteristics of partially neutralized phage have been demonstrated with mixtures in which 90 per cent. or more of the original phage population has been inactivated. Freeman's effect could not be demonstrated unless the antiserum was used in high enough concentration to inactivate at least 50 per cent. of the phage particles. The evidence, therefore, is insufficient to prove that *all* phage particles require the union of more than one antibody molecule to effect inactivation. Some certainly do, and on general grounds, e.g. comparison between the sizes of bacteriophage particles and antibody molecules, it is probable that this holds for all particles.

(C) *Aggregation Effects Manifested in Inactivation Reactions.*

Most of our discussion of the phage inactivation reaction has been concerned with relatively dilute reagents. Certain interesting complications appear when stronger reagents are used, and the original titre of undiluted phage, say 10^9, is reduced by appropriate concentrations of serum to give only a small number of plaques when plated direct. The particles "surviving" under these conditions necessarily represent those most resistant to the action of serum, and they certainly

do not constitute a representative sample of the phage population. In addition to heterogeneity of the phage population (see Section F.), two other factors have to be considered. In the first place, we have the continuing action of relatively concentrated serum on the agar surface, which has already been discussed. This results in an unduly low number of plaques, and in apparent reactivation of phage when such a mixture is diluted prior to plating. It is probably also responsible (at least in part) for the reduced diameter of the plaques observed in these circumstances.

TABLE 5.

To Show Effect of Serum on Plate and Influence of Aggregation.

Mixture	Time	Secondary dilution before plating: 0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
S. 1:30	40 mins.	*150*	*1,000*	*350*	42·5	
+ C.16 undiluted	1 hour	58·5	165	50		
	2 hours	3·5	5·5	2·5		
S. 1:30	40 mins.	78	153	34·5		
+ C.16 1:10	1 hour	25	46	9·5		
	2 hours	4·5	9	4·5		
Broth						
+ C.16 1:10	2 hours					82

Mixtures kept at 0–2°C. and diluted immediately before plating.
Italic figures estimates: the others average of two counts.

Secondly, with high concentrations of both phage and antibody, there will be opportunity for aggregation to occur. Table 5 shows the result of an experiment in which varying concentrations of phage C.16 were mixed with serum diluted 1:30 at low temperature (0–2° C.). It will be seen that the results after two hours' contact show a paradoxical relationship between phage concentration and plaque count. Undiluted phage plus serum shows fewer plaques than a tenfold dilution of phage with the same concentration of serum. Having regard to the known features of the phage-antiphage reaction, there seems to be only one possible explanation of this effect. On the "percentage law" the same proportion of "survivors" should be present in both mixtures, but in the undiluted phage mixture each "survivor" will be ten times as likely to collide with inactivated antibody-coated phage particles and take part in the formation of a lattice structure of phage and antibody. Since actual macroscopic aggregation of phage C.16 can be induced by serum, there can be no objection to applying this conception to explain an effect observed in inactivation experiments with strong reagents. In this connection it is relevant to point out that if we adopt the lattice theory put forward by Marrack (1933) to explain immunological aggregation reactions, the very fact

of the occurrence of phage aggregation with immune serum indicates that more than one antibody molecule can be attached to each phage particle. Otherwise no lattice formation would be possible.

(D) *The B. Receptors of the Bacteriophage Surface and Their Relationship to the Antigenic Groups.*

It is well known that phages are more or less specifically adsorbed to bacteria which are susceptible to their action, and it is generally accepted that this fixation is an initial and necessary stage in the process of lysis. The nature of this specific relationship between phage and susceptible bacterium has been a major object of interest to one of us for some years (Burnet, 1927, 1934a; Burnet and McKie, 1930). Indirect evidence, which has been discussed in Section 7, indicated that the bacterial component involved was the specific antigenic polysaccharide of the bacterial surface. Later work (Levine and Frisch, 1933, and Gough and Burnet, 1934) established directly that bacterial polysaccharide in the free state could combine with, and inhibit the activity of, a certain proportion of phages. Since the union was of a specific pseudo-immunological type, it was assumed that there must be certain specific atomic groupings on the bacteriophage surface ("B-receptors"), whose structure was such as to facilitate union with certain aspects of the bacterial polysaccharide structures.

The question immediately arises as to whether these B-receptors (B = related to bacterium) are the same as the antigenic groupings on the phage surface, which are responsible for union with antibody (A-receptors). Superficially they appear to be totally distinct, for the following reasons:

1. Phages falling in the same serological group may lyse widely distinct types of bacteria, e.g. in Burnet's serological group 11 there are phages which lyse coliform bacilli only (C.21), rough strains only (D.12), or a wide range of S. and R. intestinal bacteria (C.16).
2. Phages with a limited common range of activity, e.g. on smooth dysentery bacilli only may belong to distinct serological groups (D.3, D.44, D.6).
3. The activity of B-receptors can be readily modified, e.g. by passage of the phage on a heterologous strain while antigenic character remains unaltered.

There are three possible interpretations of these facts: (1) The two types of receptor are totally distinct, occupying different points on the surface of the phage particle; (2) B-receptors are portions of more complex A-receptors, variation in the B. aspect being insufficient to cause any detectable antigenic change; (3) The whole phage particle surface is active in both respects without there being any spatial differentiation of A. and B. receptors.

It is necessary to choose from these alternatives the hypothesis which is of most value in accounting for the detailed resemblance between inactivation of phage by antiserum and P.I.A.

In Section 8 (B), an experiment was described to show that the reactivity of phage with P.I.A. could be destroyed by a sub-inactivating amount of immune serum. The possibility of a selection effect having been excluded experimentally, this result appears to have important implications in regard to the surface structure of the phage particle.

(1) It indicates that more than one antibody molecule must unite to the phage particle before it is inactivated.

(2) The modified phage particle can still initiate lysis, therefore there are still free B-receptors, and since larger amounts of antibody inactivate them normally, there are also free A-receptors.

(3) At least one B-receptor has been rendered unavailable on each modified phage particle, since it cannot now be inactivated by soluble P.I.A., and since other B-receptors are still available, the one or ones rendered unavailable must be *those which are particularly reactive toward P.I.A.*

(4) The A-receptors which have rapidly united with the small amount of antibody added must be, on the average, more reactive toward antibody than those receptors which have remained free.

(5) From this it appears that union of antibody with the most reactive A-receptor of a phage particle automatically blocks the most reactive B-receptor. Unless there is some quite unknown process involved, this blocking will result from the attached antibody molecule covering, or partly covering the B-receptor concerned; in other words, the A. and B. receptors involved are spatially contiguous, and may be regarded as two aspects of a single complex group. Several such groups are probably distributed over the surface of each phage, their reactivity or avidity varying from one to another, particularly in regard to B-receptor activity, but also in regard to the ease with which antibody can be combined. An avid grouping will combine more readily either with P.I.A. or antibody than a less avid one, but the difference between the two will be more striking in regard to P.I.A. than to serum. With small concentrations of serum there is a sufficiently preferential union of antibody to the more avid complexes to leave a population of phage particles almost lacking in ability to combine with P.I.A., but yet not significantly more resistant to higher concentrations of immune serum than an unmodified population. The physicochemical basis of differing reactivity in this sense is unknown, but there are numerous analogous phenomena recorded, particularly in regard to avidity differences in toxin-antitoxin reactions.

It may make this conception clearer to point out how closely analogous are the structures postulated on the one hand for the bacterial surface, and on the other for the bacteriophage surface. In each case the specific antigen is represented by a complex molecular pattern, certain portions of which are responsible for other

activities. On the bacterial surface these portions include those by which phage union is effected, and it is probable that they also include the "active patches" postulated by Quastel (1928) in his theory of enzyme activity at bacterial surfaces. On the bacteriophage, the subordinate portion forms the B-receptor.

We feel that while there is no doubt whatever of the existence on the phage particle surface of the properties which we have ascribed to A. and B. receptors, the evidence that these are disposed over the surface in the form of discrete active patches, each of which includes A. and B. qualities, is less cogent, and the hypothesis may require modification in the light of future work. The hypothesis, however, affords the only simple explanation of the phenomena described for phage H, and fits in well with such facts as the existence of partly neutralized phage, and the detailed similarity of the inactivating effects of immune serum and of P.I.A.

(E) *Influence of Serum on the Power of Phage to "Coat" Susceptible Bacteria.*

In the course of work on the agglutination of phage-coated bacteria, Burnet (1933a) found that phage C.16 inactivated by formalin still retained its capacity to unite with susceptible bacteria and render them agglutinable by antiphage serum. This suggested that a similar technique might be applied to the study of phage particles inactivated either by immune serum or by P.I.A.

TABLE 6.

Coating Power of Phage C.16 treated with 1:100 Serum under Different Conditions.

Treatment of phage	Percentage of active phage surviving	Agglutination	
		Antiphage 1: 20	Normal Saline
1. 1: 100 serum at 0° immediate	nearly 100%	+ + +	+ +
2. " " 0° for 1 hour	50% (approx.)	+ + ±	+
3. " " 36° " "	0·68%	+	—
4. " " 45° " "	0·48%	+	—
Phage undiluted (9×10^9)	100%	+ + + +	+
Phage diluted 1: 10	10%	+ + +	±
Phage diluted 3: 100	3%	—	—
Phage diluted 1: 100	1%	—	—

If we make successive dilutions of an active phage filtrate titre 5×10^9, or slightly more, in broth, and test their power to coat a standard amount of washed Y.S., we find that the power is lost completely when the titre falls below 2×10^8. There is a sharp change between about 6×10^8, which coats well, and 2×10^8, which shows no demonstrable effect. The plan of the experiments with antiphage serum and P.I.A. was to add the inactivating agent to 5 c.c. of undiluted phage and allow it to react for a suitable period. One drop of a dense emulsion of Y.S. bacteria was added to give a content of about 250 million per c.c. After standing for two hours in the refrigerator, the tubes were centrifuged, the supernatant fluid

discarded and the deposit emulsified in 1 c.c. of saline. The bacterial suspensions so obtained were tested with antiphage serum absorbed free of bacterial agglutinins, at a dilution of 1 :20, and with saline to control spontaneous agglutination. Agglutination was allowed to proceed for two hours at 45°C. and overnight at room temperature.

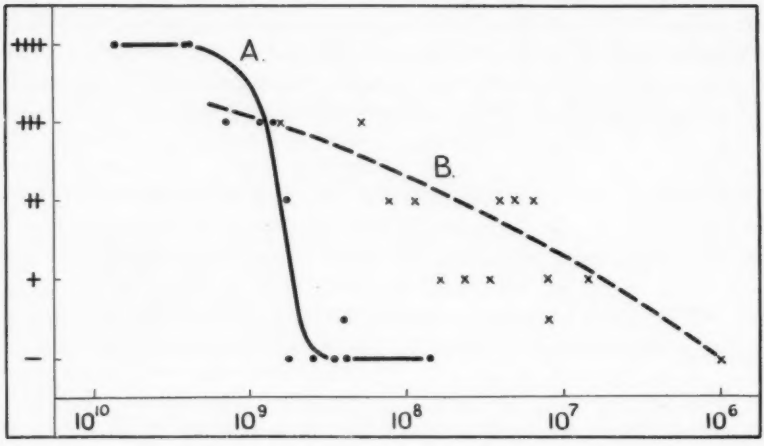

Fig. 10. The power of phage C.16 to coat susceptible bacteria and render them agglutinable by antiphage serum. A.: phage simply diluted in broth. B.: undiluted phage reduced in activity to the level shown in the abscissae by the action of antiserum, and then used for coating bacteria. Ordinates: intensity of agglutination by absorbed antiphage serum. Abscissae: numbers of viable particles per c.c. in phage preparations.

Several experiments have been made on the action of immune serum absorbed free of bacterial agglutinins in which (a) the action of different serum concentrations has been compared under the same conditions, and (b) the effect of one serum concentration acting under different conditions of temperature and time has been tested. An experiment of the second type is tabulated below (Table 6), and in Figure 10 the results of a number of experiments are combined to show the differences between the coating powers of phage simply diluted (A), and partially inactivated by serum (B).

A comparison of curves A. and B. in Figure 9 shows at once that there is no simple relationship between inactivation and loss of ability to become attached to bacteria. It appears that phage particles, which in the presence of serum are still capable of initiating lysis, may have united with sufficient antibody to diminish significantly their adsorption to bacteria, and conversely that a considerable proportion of inactivated phage particles can still unite with bacteria.

Similar experiments with phage inactivated by P.I.A. are described by Burnet and Freeman (1937). They showed that phage H. reduced to about 1–2 per cent. of its original titre showed no loss of power to coat bacteria. The agglutination was, if anything, more complete. Similarly the inactivated phage showed direct agglutination by antiphage serum as readily as the untreated phage. This clear-cut distinction between P.I.A. inactivation and serum inactivation will be discussed in the summary (Section 8, (1)).

(F) *Heterogeneity in Pure Phage Populations.*

It is a commonplace of biology that even genetically uniform populations show some degree of variation in every measurable character, and it would be foolish to expect that a pure strain phage population should be homogeneous in its resistance to inactivation by antiserum. Experimentally, C.16 and similar phages do not show any large degree of variation in respect of resistance to serum inactivation. Probably the deviation of the time-survivor curve from a logarithmic form is an indication of heterogeneity but, as can be most easily seen from serum concentration-survivor curves (e.g. Figure 3), none of the particles shows any exceptional resistance to the action of serum. With other phages there is a wider variation, the serum concentration-survivor curve of S.13, for example, is of a distinctly sigmoid form, indicating a proportion of highly resistant particles (Figure 5).

Perhaps the most striking evidence for heterogeneity amongst phage particles is provided by an experiment of Andrewes and Elford (1933). A phage-serum mixture was prepared and titrated after some hours, when the plaque count had reached a low, but only slowly falling, level. An amount of fresh phage, equal to that originally present, was now added, and titrations were made at succeeding intervals. The fall in titre of the freshly added phage was almost as rapid as the original primary fall, and far more rapid than the rate of inactivation of the small proportion of relatively resistant survivors had been at the time the fresh phage was added.

Fig. 7 shows a serum concentration-survivor curve with the rather feebly antigenic phage S.18. Here there is a considerable range of dilutions over which the serum has a small but definite inactivating effect. In the curve shown there is an indication of a step as if the population could be divided fairly sharply into very susceptible and relatively insusceptible particles. Three other curves showed a similar step, and another the same type of flat curve but without any distinct discontinuity. All these experiments were made with phage re-isolated from a single plaque.

It is safe to assume that all phages show a frequency distribution of resistance to serum. In some, such as C.16 and the highly antigenic staphylococcal phage, it is probably of a steep normal type, while in others it is more dispersed, and probably in many instances shows a skew deviation.

Heterogeneity of the phage population will of necessity complicate any attempt at simple mathematical formulation of the kinetics of the reaction, but with the more uniform types such as C.16, certain limited conclusions can be drawn from a study of the course of the reaction under different conditions.

(G) *Heterogeneity of Antibody Molecules.*

Modern writers on the nature of serum antibody agree that the antibody molecules are globulin molecules, certain superficial atomic groupings of which have been modified so that they may unite specifically with one or more of the "determinant groups" of the antigen. Even with the simplest antigens there is evidence that the population of antibody molecules in an immune serum is not a homogeneous one. Avery, Goebel and Babers (1932), for instance, immunized rabbits with an artificial antigen (carrying as its determinant group an α glucoside), obtaining an antiserum which would react with suitable antigens carrying either the homologous determinant group or the corresponding β glucoside. Absorption with the heterologous antigen removed all reactivity for this, but left reactivity toward the homologous antigen hardly diminished. In this way the antibody population was shown to be divisible into two groups, those capable of union with this heterologous antigen, and the rest. Similar findings can be multiplied, particularly from the literature on the cross reactions of immunologically related bacteria. Furth and Landsteiner (1929) and Burnet (1934b) have brought evidence that in the case of bacterial antisera the heterogeneity amongst antibody molecules is not a simple reflection of multiplicity of components in the corresponding antigens. It arises because as a rule each antibody molecule can carry modified superficial groupings corresponding only to a proportion of the number of qualitatively distinct determinant groups of the antigen molecule.

Other evidence of antibody heretogeneity may be obtained by comparing antisera obtained from the same animal, (1) in the early stages of immunization, and (2) after prolonged immunization with the same antigen. Heidelberger and Kendall (1935) showed that the antibody produced by rabbits immunized with pure crystaliine egg albumen was not homogeneous, but included much "low grade" antibody not readily precipitable by antigen. During prolonged immunization, the serum from a single animal showed progressive changes, indicative of the formation of increasing amounts of antibody capable of reacting with a larger number of chemically different groupings in the antigen molecule. It is well known that if sera are required for the immunological differentiation of related proteins, it is advisable to give only a short course of immunizing injections. The resulting sera are of much higher specificity than those obtained after a long series of injections.

Heterogeneity of antibody produced in response to bacteriophage injections in rabbits can be demonstrated in both these ways. Burnet (1933a) absorbed antiphage sera with bacteria which had been coated with homologous or related heterologous phage, and showed that the usual relation between homologous and hetero-

logous antigens obtained. Serum absorbed by the heterologous phage lost almost all its activity against this, but retained practically its full titre against the homologous phage.

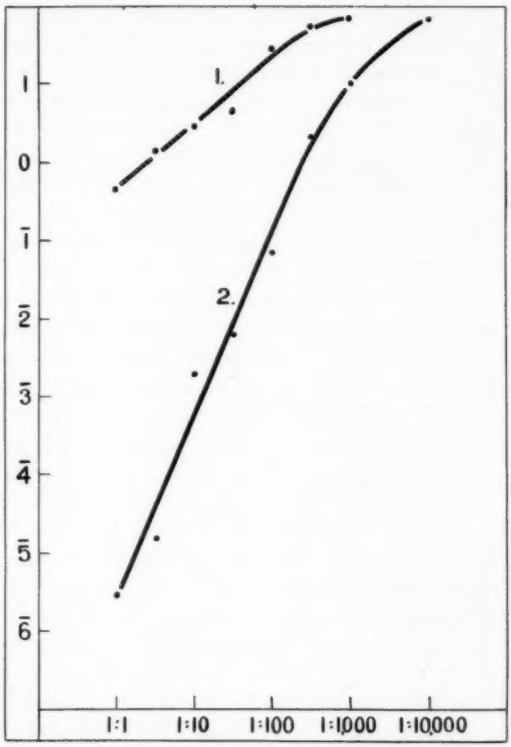

Fig. 11. Concentration survivor curves for phage L. with sera from two bleedings from the same rabbit, early (1) and late (2) in the course of immunization.

Changes in the character of an antiphage serum during the course of immunization have been observed by Keogh and Simmons (unpublished). Figure 11 shows the results obtained when sera from two bleedings of the same rabbit immunized with phage L. are compared. The curves represent the percentage reduction in plaque count obtained with varying dilutions of serum under constant conditions of time and temperature. It will be seen that while the later bleeding produces a far greater reduction when used in relatively high concentration, the difference with dilute (1 : 1,000) serum is very much less. Both sera appear to contain similar numbers of antibody molecules, but in the earlier one these are capable of inactivat-

ing a smaller proportion of the phage particles than are those from the later bleeding. The resemblance to the findings of Heidelberger and Kendall (1935) is obvious.

Rather similar evidence of antibody differences is mentioned by Andrewes and Elford (1933). With one batch of serum against C.36 a certain degree of dissociation could be demonstrated if mixtures were diluted soon after being made. With another more potent anti-C.36 serum the effect was not demonstrable. Presumably the first serum contained on the average more "low grade" antibody molecules than the second.

(H) *The Quantitative Aspects of Phage Neutralization.*

In the last analysis the reaction between phage and antibody is the result of collisions between discrete entities in an aqueous medium, and should be capable of quantitative description in terms of the kinetic theory and the laws of mass action. It will be apparent from the preceding sections, however, that there are many complicating factors to be considered, which will certainly prohibit any simple formulation of the reaction. In general, equilibrium conditions are more suitable for discussion than reaction velocities, but in phage work very little attention has been paid to the question as to whether a true equilibrium condition is ever attained. On general grounds it seems likely that with very weak serum and a sufficiently long period of contact, evidence of a true equilibrium might be obtained, but it would probably be difficult to avoid spontaneous inactivation of phage. With stronger serum, any residual activity would certainly be due to the more resistant particles, and no conclusions as to the nature of the processes responsible for the apparent equilibrium could be drawn. In the subsequent discussion of virus neutralization by serum, equilibrium considerations are the dominant feature, and it is unlikely that there are wholly distinct processes at work in the two cases. The experiment shown in Table 4 makes it clear that union of antibody with phage C.16 is reversible, and that dissociation can be demonstrated if an alternative agent capable of uniting with the antibody is added. If dissociation occurs in the presence of phage-specific soluble substance, it must equally occur in its absence. It is only the impracticability of experimental studies on the point that has left the position obscure.

Under the circumstances, any attempt at approximate mathematical formulation can be applied only to the *rate* of inactivation. To simplify conditions as far as possible, we may assume (a) that the phage population is homogeneous, (b) that in all reaction systems the amount of antibody is so large that union with phage will not result in any significant lowering of antibody concentration, (c) that the reaction is completely irreversible, and (d) that the antibody molecules can be regarded as homogeneous.

In the first place, it is necessary to discuss briefly the significance of the "percentage law". This is simply that in the systems being considered the number of

antibody molecules per unit volume is so much greater than the number of phage particles that there is no competition between phage particles for the available antibody. In Andrewes and Elford's words, we can concentrate "on the fate of each individual phage particle in its sea of antiserum". Where conditions are such that this description is inapplicable, the "percentage law" breaks down.

Confining ourselves to systems in which the "percentage law" will apply, the *proportion* of surviving particles is independent of the phage concentration, and provided we deal with proportions and not absolute numbers of survivors, we can omit the concentration of phage from discussion, regarding it as constant throughout. Under these conditions, the rate of the reaction should be directly proportional to the concentration of serum (C) if a simple bimolecular reaction occurs, or to a higher power of C. if the reaction is more complicated. It is extremely unlikely that the reaction is of any higher order, and we may take the simplest condition first. The usual formula for bimolecular reactions, $k=\frac{1}{t}\cdot\frac{1}{a-c}\log.\frac{(a-x)c}{(c-x)a}$ where a is the initial concentration of phage, x the amount inactivated in time t, and c the concentration of antibody, becomes, when c is assumed very large compared with a or x, $k=\frac{1}{t}\cdot\frac{1}{-c}\log.\frac{a-x}{a}$ or $kc=\frac{1}{t}\log.\frac{a}{a-x}=\frac{1}{t}\log.\frac{1}{P}$ when P is the proportion of surviving phage particles at time t. When plotted semilogarithmically, the course of inactivation should be represented by a straight line. This result is what would be expected if a single antibody molecule could inactivate a phage particle to which it was attached. But we have already given evidence that more than one antibody molecule is necessary for inactivation in most cases at least, and in practice there is no way in which the course of the primary reaction, which may be represented $P + A = PA$, can be followed. What is observed is probably the resultant of a series of reactions: $P + A \rightarrow PA$, $PA + A \rightarrow PA_2$, ... $PA_{n-1} + A \rightarrow PA_n$. Of the various combinations, those containing less than a certain number of antibody molecules will retain lytic activity, while those with more than this will be inactive. Assuming that serum concentration remains constant, and that the various active groups on the phage particle surface are of equal reactivity, each of these reactions will proceed at the same rate for similar concentrations of P (free or combined, as the case may be); but it is only the final reaction $PA_{n-1} + A \rightarrow PA_n$ in which an active particle is converted into an inactive one which determines the experimentally demonstrable course of the reaction. The rate determined for this reaction will be slower than that of any of the constituent reactions, since it can be measured only in terms of the total number of phage particles having less than n antibody molecules, not in terms of those actually carrying $n - 1$ antibody molecules. To a sufficient approximation the velocity will be $\frac{k}{n}$ over that part of the reaction which follows an approximately logarithmic course.

Considering the relative inaccuracy of the titration methods and the various complicating factors which have been mentioned earlier, the differences between the results to be expected from quantitative experiments were a simple one to one union sufficient to inactivate, and those when two or more antibody molecules are required, are probably insignificant, except when the reaction is very slow. When small concentrations of serum are used at low temperature, there is a well marked lag period before the approximately logarithmic decrease in plaque counts commences. With higher serum concentrations and the same experimental conditions, there is no such lag, so that its origin must be sought in the quantitative relationships of the reagents. If more than one antibody molecule is required for inactivation, it is easy to see that with very dilute serum there will necessarily be a considerable period elapsing before a sufficient proportion of phage particles carrying subinactivating amounts of antibody will accumulate to allow significant inactivation to be observed. The experimental evidence for such a lag period has been described in Section A (iii), and this phenomenon provides further evidence for the view that more than one antibody molecule is required for inactivation.

So many complicating and qualifying factors have now been enumerated that it seems almost a hopeless task to attempt to determine whether the relation $kC=\frac{1}{t}\log.\frac{1}{P}$ is a valid description of the course of the reaction. All that can be done is to eliminate the complications as far as possible and see whether over a limited range of conditions the reaction velocities are of the approximate order of magnitude required by the formula. To some extent, the complicating factors can be avoided or minimized as follows:

(i) *Heterogeneity of phage population.* If the frequency distribution of resistance to serum is of approximately normal form, the standard rate of inactivation for any set of conditions should be the rate at the time when 50 per cent. of the phage has been inactivated. A tangent to the experimental curve at this point will theoretically therefore give the most satisfactory value for the reaction velocity. Except with very dilute serum, however, it is difficult to obtain accurate results within the very short time period available, and if relatively strong phage is used, errors in making the high dilution required to give countable plaques may be important.

(ii) *Aggregation phenomena.* These become important only when high concentrations of phage are present, and can be avoided by working with very dilute phage.

(iii) *Continuing action of serum on the agar surface.* This is naturally of much greater importance with strong serum than with dilute, and can be rendered insignificant if the reacting mixtures are always diluted so that the concentration of serum in the material plated is 1 : 1,000 or less. If any important degree of dissociation occurs, this procedure would be unjustifiable, but with a phage like C.16 this complication can be neglected.

Experiments carried out under these restricted conditions on the inactivation of phage C.16 by various concentrations of a given homologous antiserum at low temperature (1–3° C.) have shown that there is a fair approximation to the formula $\frac{\frac{1}{t}\log.\frac{1}{P}}{C}=k$. The values of $\frac{1}{t}\log.\frac{1}{P}$ are given for concentration of serum from 1 : 30 to 1 : 30,000 in Table 7.

TABLE 7.

Values for $\frac{1}{t}$ *log.* $\frac{1}{P}\cdot\frac{1}{C}$ *for Various Serum Dilutions at 0–4° C.*

Serum Dilution	$\frac{1}{t}\log.\frac{1}{P}\cdot\frac{1}{C}$			
1 : 30	63	87	88	84
1 : 50	49	54		
1 : 100	45	36		
1 : 300	34	49	45	
1 : 1,000	34	47	43	53
1 : 3,000	44			
1 : 10,000*	70			
1 : 30,000*	82			

Table 7 combines the results of all satisfactory experiments done according to the technique described in the text. The two lowest concentrations (*) required weeks to show inactivation, and were kept in the refrigerator at a temperature a few degrees higher than the ice bath temperature used in the other experiments.

t. in hours.

Except for the strongest concentration given (1 : 30), in which a certain amount of aggregation may have occurred, the results all give a value for k which is not far removed from 45. The highest dilutions of serum, 1 : 10,000 and 1 : 30,000, were used in an experiment extending over some weeks, and the tubes were left in the refrigerator between platings at a temperature between 4° and 6° C., i.e. distinctly higher than the ice bath temperature used for the shorter periods.

At 37° C. the value of k is much greater, and again approximately constant when estimations of serum activity are made under the restricted conditions which have been prescribed. Values for successive serum dilutions were: Serum 1 : 100, $k = 1{,}000$; 1 : 300, $k = 826$; 1 : 1,000, $k = 1{,}150$; 1 : 3,000, $k = 740$.

Tests of the same batches of serum and phage at a temperature of 45° C. gave the following values for k in two experiments:

Experiment 1. Serum 1 : 300, $k = 1{,}260$; 1 : 1,000, $k = 1{,}616$; 1 : 3,000, $k = 1{,}150$.

Experiment 2. Serum 1 : 300, $k = 1{,}430$; 1 : 1,000, $k = 1{,}500$; 1 : 3,000, $k = 1{,}150$.

(I) *Summary.*

We may conclude that in all essential features the phage-antibody reaction resembles other antigen antibody reactions. Its peculiarities depend in part on

the size and number of the particles concerned, in part on the special experimental method used to study the inactivating effect of immune serum.

With the larger bacteriophages like C.16, macroscopic aggregation reactions can be observed if sufficiently high titre material is available, and it has been shown by absorption experiments that these reactions are due to the same antibody as is responsible for phage inactivation. With smaller phages or suspensions of lower titre, aggregation cannot be observed for simple reasons of size and number, which have been discussed by Merrill (1936).

The nature of the phage inactivation by immune serum has been discussed on a basis of the hypothesis that phage particles carry on their surfaces a number of active groups, each of which has a dual specificity, (a) toward antibody, (b) toward certain components of the surface of the susceptible bacterium.

The essential feature of the reaction is a simple union of antibody molecules to the active centres of the phage surface. This union is comparatively slow, but probably no more so than any other antigen-antibody reaction. It must be remembered that while in most immunological reactions the reaction may be regarded as complete when 99 per cent. of the antigen is combined, with phage it is easy to titrate 1/1,000,000 of the original activity, and the reaction is usually studied with much more dilute reagents than would be used in ordinary *in vitro* immunological tests. The rate of union increases with rise of temperature, and with dilute reagents is directly proportional to the serum concentration. When the complications due to aggregation and the continuing action of serum on the surface of the titration plate are eliminated, the rate of inactivation, after a period of lag, which is only noticeable with very dilute sera, takes up a steady value which, within the limits of accuracy of the experiments, is directly proportional to the concentration of serum. This uniform rate of inactivation persists only as long as the proportion of surviving phage does not fall below about 1 p.c. With greater reduction, the heterogeneity of the phage particles becomes evident, and the rate of inactivation slows down rapidly.

No evidence of reversibility of the phage antiphage union can be obtained from dilution experiments, but by indirect methods a certain degree of reversibility is demonstrable. Theoretically, an equilibrium between phage, serum and inactivated phage should develop, but technical difficulties have prevented any demonstration that such is the case.

A variety of evidence has been given that "partially neutralized" phage particles can exist, i.e. that more than one antibody molecule must be attached to a phage particle before it is inactivated. Evidence has also been adduced that phage particles inactivated by serum may still retain ability to become attached to the bacterial surface. In all probability there is no single mechanism by which union of antibody to phage prevents the initiation of lysis. Our knowledge of the details of lysis by phage is very slight. It is known that specific adsorption takes place, and that when phage multiplication occurs the descendant particles are retained

in or on the bacterium until the moment of actual lysis, but the processes occurring between adsorption and the commencement of multiplication which are of particular importance in the present connection, are completely unknown. It is tempting to discuss inactivation by antibody wholly in terms of interference with adsorption to the bacterial surface, which, as we have shown, is the one aspect of phage activity about which a good deal is known. But the demonstration in Section 8E that inactivated phage can still be adsorbed to bacteria makes it clear that this cannot be the main factor. It seems best to regard the action of antibody attached to phage particles as diminishing the *probability* of their effecting lysis within the time available, to a degree proportional to the number of antibody molecules attached to each particle. The factors responsible for this diminished probability may be (1) actual steric blocking of B-receptors, especially those on the most reactive portions of the phage surface. If this blocking is complete, no lysis can occur, but in all probability many particles which fail to produce plaques in neutralization experiments are by no means completely coated in this way. Even with these, however, if any B-receptors are blocked there will be a correspondingly greater average period before effective contact with the bacterial surface takes place. (2) Hindrance to the entry of an adsorbed phage particle into the bacterium. If a phage particle carrying one or more attached antibody molecules becomes specifically adsorbed to a susceptible bacterium, it seems reasonable to consider that the more antibody there is present, the more difficult it will be for the phage particle to enter the bacterium and commence to multiply. It is possible that such phage particles cannot enter until they are freed from antibody by the occurrence of the normal spontaneous dissociation which has been postulated, or in some other manner. In any case, the initiation of phage multiplication and lysis will be delayed. (3) Aggregation. It is obvious that if phage aggregates are plated with susceptible bacteria, only one plaque can possibly develop from each aggregate. In any case, a phage particle linked to others by antibody molecules will be unlikely to make effective contact with a bacterium, and if it does, will be even more unlikely to enter the bacterium unless the aggregate is disintegrated. As we have discussed earlier, aggregation will occur only when the concentration of phage is sufficiently high and adequate time is allowed. With dilute phage it probably plays no significant part.

The detailed experimental work on which these conclusions are based has been carried out almost entirely with phage C.16, and to some extent the discussion is only valid for phages of this particular type. It has been shown (Burnet, 1933d) that C.16 is a typical member of the commonest serological group of dysentery-coli phages, and as such is probably the most suitable phage to serve as a representative type. Sufficient work has, however, been done with small particle (S.13) and intermediate (C.36, S.18) phages to indicate that broadly the same results are obtained, and it is unlikely that detailed studies of these would provide evidence of different processes being involved in their inactivation by immune serum.

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Chapter IV.

IMMUNOLOGY OF PLANT VIRUSES.

Study of plant viruses by immunological methods is a recent development, but is becoming of considerable importance, and seems likely to provide the most satisfactory criterion for classification of these viruses, just as it has with bacteriophages. In this review we are not concerned with such problems as the differentiation of plant virus types, or the analysis of multiple virus infections such as some of the potato disease syndromes, by serological methods. Our only interest is to examine what has been recorded concerning the nature of the serum-virus interaction and its influence on the infectivity of virus suspensions for the susceptible plant in the light of the work which has been done in the fields of animal-pathogenic viruses and bacteriophages. For this purpose the available material is concerned almost entirely with tobacco mosaic.

The infective agent of this disease is at present unique amongst viruses, in that it has been obtained in the form of a crystalline protein by Stanley (1935). There seems to be no reason to doubt that the crystals prepared by Stanley's methods actually represent orderly aggregates of the virus particles responsible for the disease. Ultrafiltration studies with Elford's gradocol membranes have shown that the virus particles concerned are of the same size as the smallest animal viruses and bacteriophages—about 10 mμ. This is considerably larger than most protein molecules, but smaller than the crystallizable protein haemocyanin.

Bawden *et al.* (1937) have recently published a preliminary account of the interesting physicochemical properties of the purified agent. They interpret its behaviour as indicating that the unit particles are in the form of slender rods, thus differing sharply from any other virus or soluble protein which has been studied in this respect.

There has been a tendency to construe the fact that tobacco mosaic virus can be isolated as a pure protein as indicating that it is not living. At this level of organization, discussion of whether an agent is living or not is very difficult, but it is of particular interest that mosaic virus shows a marked tendency to produce mutations whose peculiarities are indefinitely transmissible. At least 20 recognizably different varieties of the infection are known, and all those tested have been found to be of the same particle size (Thornberry, 1935), and very similar or identical by serological tests (Chester, 1935). Ability to undergo mutations which are genetically transmissible is often considered the most characteristically vital aspect of a living organism.

Chester (1935a) has pointed out one implication of Stanley's work that is of importance in the present connection. This is that, although the purified virus is highly infective, its infectivity is enormously less than would be calculated for the material if every "molecule" or virus particle could initiate infection. Compared

with virus infections of animals or even with bacteriophage, the tissues of the infected plant contain a greater mass of actual virus material, and having regard to the small size of the infective particle, a far greater number of virus particles.

The potato virus *x*, the only other virus that has been extensively investigated, shows the same discrepancy between amount of virus and infectivity.

Purdy (1929) showed that sap from tobacco plants infected with mosaic contained an antigen which was not present in normal sap. Rabbits inoculated with the virus-containing material produced a serum which could be absorbed free of antibodies to normal plant juice constituents, and then produced a specific precipitate when mixed with sap from infected plants. The serum had also some power to destroy the infectivity of the virus as tested by inoculation of susceptible tobacco plants.

Subsequent work has shown that the antigen involved in this reaction is found only in plants demonstrably infected by the virus, and that it is identical whatever host plant is used for the propagation of the virus. The intensity of the reaction is proportional to the infectivity of the virus-containing material. With potato *x* virus, Bawden (1935) found that the amount of antigen determined by the optimal proportions titration method was closely proportional to the infectivity. Methods of purification which concentrate the infectivity of a preparation likewise increase the proportion of precipitable antigen, and conversely, exposure to heat sufficient to inactivate the virus also completely destroys reactivity with serum (Chester, 1935b). Most chemical inactivating agents, with the exception of formaldehyde, destroy serological reactivity as well. Formalinized virus, though non-infective, reacts with serum, and at least in the case of *x* virus, retains its antigenicity. Studies with graded collodion membranes show that the antigenic and the infective particles of a preparation are of the same size. Finally, antisera, which cause specific precipitation, also cause inactivation of the virus as judged by quantitative tests of infectivity. There is available, therefore, almost absolute evidence that the specific antigen and the virus are identical, and in the discussion it will be assumed that such is the case.

As regards *in vitro* reactions, there is no reason to regard the tobacco virus protein as differing significantly from any other actively antigenic protein. A rapid typical immune precipitation occurs, showing the usual zone of optimal flocculation when the proportions of the reagents are varied. Complement fixation can be demonstrated with the usual technique (Chester, 1935a). Specific anaphylactic reactions, however, cannot be elicited (Chester, 1936), although both crude and purified virus contain easily detectable amounts of normal plant protein which is an active anaphylactogenic agent. Chester considers that the reactions of the crystallized virus with guinea-pig uterus, sensitized by normal tobacco protein, indicate that the crystals are contaminated with normal protein, rather than that they possess antigenic determinants in common. The non-reactivity of the virus protein he would ascribe to its very high molecular weight and low diffusibility.

Of chief interest in relation to the analogous phenomena with animal viruses and bacteriophages is the reduction in infectivity produced by mixing serum with virus. In recent years, titrations of infectivity have nearly all been made by a method similar in principle to plaque counting methods with phage, or the chorioallantoic membrane technique with certain animal viruses. The preparations to be tested are spread by some suitable method over the surface of a standard number of leaves of a suitable test plant. After one minute's contact, the leaf

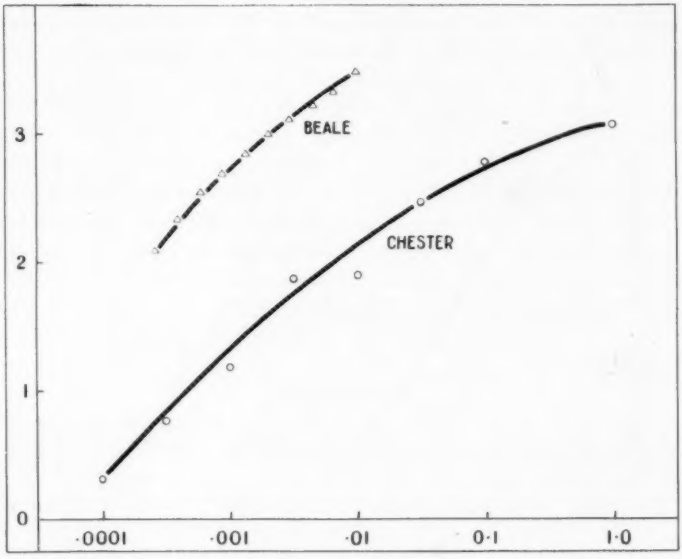

Fig. 12. Relation between concentration of mosaic virus and the average number of necrotic foci produced on susceptible leaves. Log. numbers of foci plotted against virus concentrations. Data from Youden, Beale and Guthrie (1935).

surface is washed with water from a wash bottle. After three or four days under standard conditions of temperature and illumination, necrotic foci appear on the leaves whose number is related to, but not directly proportional to, the concentration of virus used. With dilute virus there is a close proportionality, but as the concentration increases, proportionally smaller numbers of foci appear. Figure 12, taken from data of Youden, Beale and Guthrie (1935) shows the relation between virus concentration and the counts of necrotic foci. The number of foci produced with high dilutions of virus is much lower than the total number of virus particles which can be calculated to be present in the inoculum from other considerations. Best (1936) calculates that a number of virus particles of the order

10^9 are required to produce a single lesion. The conditions are very different therefore from those obtaining in work with bacteriophages or animal viruses on the chorioallantoic membrane. With one phage Schlesinger (1933) calculated that

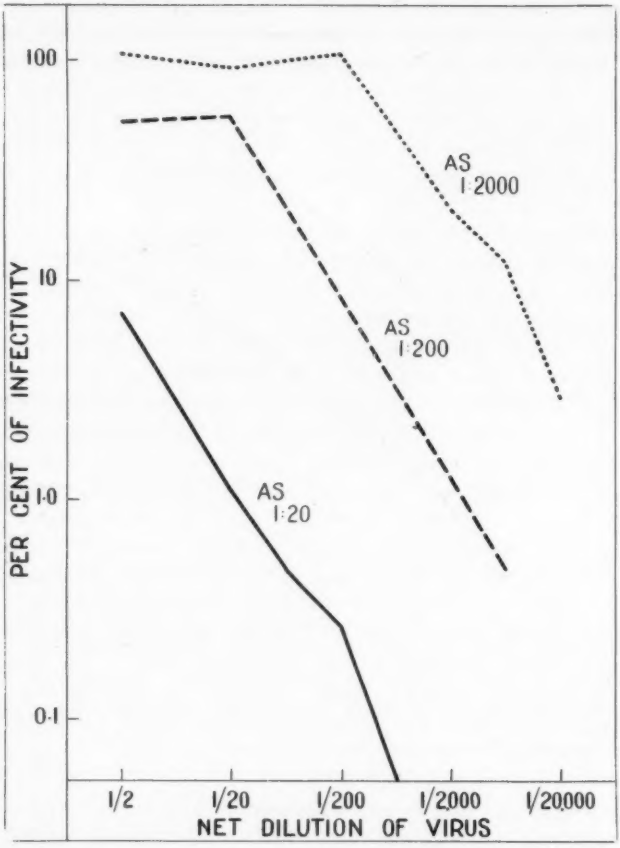

Fig. 13. To show the infectivity of different mixtures of mosaic virus and antiserum. The mixtures were tested by leaf surface inoculation, and the infectivity is shown in terms of the percentage of necrotic foci produced relative to the number obtained with equivalent normal serum-virus mixtures. Figure redrawn from Chester (1934).

50–70 per cent. of the particles observable under the ultramicroscope were capable of producing plaques. Burnet (1936) has presented evidence that probably about 50 per cent. of the virus particles of egg passage influenza virus produce a visible focal lesion on the egg membrane.

The proportion of lesions produced by mixtures in which the concentrations of serum and virus were varied has been studied by Chester (1934), and Figure 13 is reproduced from his paper. It shows at once that nothing corresponding to the percentage law holds with this system. A dilute serum shows no effect unless the virus is sufficiently diluted, and to a rough approximation the first indication of activity occurs when the virus and serum concentrations are at a certain ratio. A tenfold concentration of serum inactivates approximately ten times as much virus as unit concentration.

The interpretation of this result depends on the fact that we are dealing with an enormously higher concentration of antigen than in animal virus work and, in the system studied, antibody and antigen molecules are present in almost equivalent numbers. With animal viruses and bacteriophages we have the individual virus particles of relatively low avidity for antibody floating in a (metaphorical) sea of antibody. With tobacco mosaic virus the system is far more concentrated, typical aggregation by lattice formation occurs, and with the lower concentrations of serum practically the whole of the antibody is bound by an equivalent amount of antigen, leaving any remaining virus able to produce its normal infective titre. The virus particles are excellent antigens, but probably no more so than typical phage particles,—the difference in their reactions is to be ascribed almost entirely to the greater concentration of the antigen allowing lattice formation and precipitation, and to the very low infectivity of the material.

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Chapter V.

GENERAL CONSIDERATIONS ON THE REACTIONS BETWEEN ANIMAL-PATHOGENIC VIRUSES AND HOMOLOGOUS ANTIBODIES.

From the time of Jenner onward, immunological aspects have tended to dominate all studies of the viruses pathogenic for man or animals. The immediately obvious fact of post-infection immunity has at all times stimulated the hope of imitating this result of infection without its attendant risks. The experimental study of viruses begins with the classical researches of Pasteur on hydrophobia, researches initiated with the direct aim of finding a method of prevention or cure, and eventually providing the first successful application of immunological methods to a practical need. With the appearance of the conception of humoral immunity at the end of the nineteenth century, attempts were naturally made to ascribe immunity to virus diseases also to humoral factors. Vaccinia virus was, and has continued to be, the favourite object for research on the more theoretical aspects of immunity to virus infections, and serious study of the humoral factor may be said to have begun with the work of Béclère, Chambon and Ménard in 1898. Their first paper dealt with the passive transfer of immunity to heifers by the injection of serum from immunized animals. The nature of the mechanism by which such immunity was transferred was the subject of a second paper (1899), in which the *in vitro* reaction between virus and immune serum was described. They conclude their paper with the observation: "On ne saurait encore affirmer si cette substance agit directement sur les agents infectieux comme virulicide, ou si elle agit comme un stimulant sur les cellules de l'organisme." At this early stage they had thus formulated the problem which has continued to provide a subject of controversy up to the present time.

As an introduction to the sections dealing with the immunological reactions of particular viruses, we may briefly review the development of ideas in regard to the neutralization of vaccinia virus by immune serum. Most early investigators considered that the reduction or complete removal of infectivity, which resulted when immune serum was mixed with active virus, was due to destruction of the virus by antibody. From analogy with the action of immune bacteriolysins on the cholera vibrio, the virus was supposed to be killed and probably dissolved. Later work, however, showed that so-called neutral or inactivated mixtures could be readily re-activated by various procedures, the simplest being by dilution of the mixture in some indifferent fluid (Andrewes, 1928, 1930). Under the ordinary conditions of experiment, both Andrewes (1928) and Long and Olitsky (1930) considered that no evidence could be obtained that any *in vitro* union of virus and

antibody occurred. Later, Andrewes (1930) produced evidence that a stable union could take place, but required some days of contact between the reagents before it was demonstrable. Wilson Smith (1930) found that protective antibody could be removed from an antiserum by absorption with virus-containing tissues. The experiment was not always successful, and though it indicated that antibody and virus could, under certain circumstances, combine, it did not prove that all neutralization by serum was a result of virus-antibody union. Sabin (1935) takes as the starting point of his discussion of the problem the fact that by high speed centrifugation active vaccinia virus may be quantitatively recovered from a "neutral" virus-antiserum mixture. In his view, the antiserum acts primarily on the susceptible cells, rendering them resistant to the infective action of the virus.

With the development of methods for the experimental study of other viruses, similar findings have been recorded for several viruses other than vaccinia, and it seems likely that there is an underlying unity amongst all virus-antiserum reactions.

The development of a "pock counting" technique for the titration of viruses capable of growth on the chorioallantois of the developing egg provided an opportunity for a more quantitative study of neutralization by immune serum. Such experiments have been carried out in this laboratory with the viruses of vaccinia, influenza, laryngo-tracheitis of fowls, louping ill, rabbit myxomatosis and Newcastle disease of fowls. The presentation of the results obtained in these studies, and their discussion in comparison with the results obtained by other methods, comprise the main portion of the present work.

In order to prevent undue repetition, and to make the aims and results of the various types of experiment used intelligible, it has seemed best to precede the detailed account with a general *a priori* discussion of the probable nature of the various interactions between virus, immune serum and susceptible living cells.

Throughout the work we shall adopt the point of view that the immunological reactions of viruses must be co-ordinated with the general body of immunological theory, the main outlines of which can now be regarded as fixed (see Chapter II). The chief reason for detailed study of the virus reactions will then be to determine in what ways the particular characteristics of viruses, such as their particle size, their concentration in the infective material used, and their strictly intracellular habitat, are concerned in giving rise to the immunological phenomena which are susceptible to experimental study. These phenomena can be grouped as follows:

(1) The development of active immunity after infection, or following artificial methods of immunization. This aspect of virus immunity, together with the related questions of modified reactivity in immune or partially immune animals (accelerated reactions, allergic responses to killed virus, etc.) will only be touched on incidentally.

(2) The action of immune serum in diminishing or destroying the infectivity of virus with which it is brought in contact.

(3) *In vitro* reactions between virus and immune serum, which do not require inoculation of a susceptible animal for their demonstration, including agglutination and precipitation reactions and complement fixation.

1. NEUTRALIZATION REACTIONS.

In regard to the second group—neutralization reactions—we may confine the discussion to virus-serum mixtures which are inoculated into the susceptible tissue shortly after mixing, and omit for the present consideration of changes requiring hours or days to be manifested. All authors are agreed that the greater part of the neutralizing effect of serum is apparent immediately after mixing. There is very little increase in progressive inactivation with time, such as is characteristic of the bacteriophage-antiserum reaction. Secondly, there is general agreement that a considerable degree of reactivation—some authors would say quantitative reactivation—occurs when mixtures of virus and serum are diluted in saline or subjected to centrifugation at high speed with re-suspension of the deposited virus in saline. There are only two possible hypotheses to account for these facts. Either no union occurs at all *in vitro*, or the union between virus and antibody which is responsible for the inactivation is readily reversible. On the first hypothesis, antibody must act, as Sabin believes, on the susceptible cell. The primary objection to this view is its incompatibility with the general body of immunological knowledge. Unless very cogent experimental evidence to the contrary is brought forward, we must believe that virus antibodies are of the same general character as those provoked by all other types of antigen, including, as we have shown, plant viruses and bacteriophages. Serum antibody is made up of globulin molecules, certain active areas of which are modified to make specific union with antigenic determinant groups on the surface of the antigenic molecule or particle (Marrack). It is at least the most *economical* hypothesis that the effects produced by antibody are due to union of antigen and antibody. In all other branches of immunology, this hypothesis has been uniformly successful, and it is our object to show that it is equally applicable in the sphere of virus immunology.

We shall take as a working hypothesis that *in vitro* there is a reversible union of antibody to determinant groups on the virus particle, and that such union is responsible for the reduction in infectivity observed. If we make certain simplifying assumptions, it is possible to deduce for an "ideal case" what are the quantitative consequences of such an hypothesis. The degree to which the experimental results correspond to, or deviate from, such deductions will then provide evidence both in regard to the validity of the basic hypothesis and to indicate in what respects our simplifying assumptions are deviated from in any particular case. For our ideal case we assume:

(1) That union between antibody and the determinant groups is freely reversible, and follows the ordinary laws of reversible chemical reactions.

(2) That the potential viability of all virus particles is unaffected by union with antibody.

(3) That the populations of virus particles and antibody molecules are composed of uniform individuals.

(4) That the "survival" or "inactivation" of a given virus particle is determined by the amount of antibody combined with it at the moment of effective contact with the susceptible cell.

If the reaction is of the type in which one antibody molecule unites with one active area on the surface of a virus particle, the most convenient starting point is Langmuir's formula for freely reversible adsorptions:

$$(1) \quad kC = \frac{y}{100 - y}$$

where C is the concentration of antibody, and y is the antibody fixed expressed as a percentage of the total antibody the antigenic virus surface can accommodate. If we assume the simplest possible condition, that one virus particle can combine with only one antibody molecule, and is thereby inactivated, the formula becomes

$$(2) \quad kC = \frac{\text{Percentage of virus particles inactivated}}{\text{Percentage of virus particles surviving}} = \frac{100 - P}{P}$$

where P is the percentage of survivors. Such an assumption, however, is quite unwarranted. Each virus particle can undoubtedly combine with more than one antibody molecule, and the formula cannot be directly applicable. Having regard to the relatively large experimental error of all our titrations, it may be legitimate to make certain simplifying assumptions which will still allow this formula to represent the conditions to a sufficient approximation.

Suppose that within the range of concentrations studied q antibody molecules can unite with each virus particle, thereby inactivating it, while $q - 1$ are insufficient to do so. Then when the proportion P of survivors is small, practically all survivors will have $q - 1$ antibody molecules attached,

$$\text{and} \quad (3) \quad kC = \frac{(100 - P)q}{P(q - 1)}$$

$$k \frac{q - 1}{q} \cdot C = \frac{100 - P}{P}$$

$$k' . C = \frac{100 - P}{P}$$

At extremes of concentration such an assumption will certainly be invalid, but it is not unreasonable to suppose that in the range chiefly studied, where there are from 0·1 p.c. to 20 p.c. of survivors, there is, on the average, a difference of only one antibody molecule between the numbers united to "surviving" and "inactivated" virus particles respectively.

When the percentage of survivors is less than 20 p.c., a further rough approximation reduces the formula to $k'C = \frac{100}{P}$ (since a difference between 80 p.c. and and 100 p.c. in the numerator is within the range of experimental error), or C. P = constant.

An identical formula to that of Langmuir can be derived from the laws of mass action. It appears preferable to consider the reaction as a reversible adsorption, since fewer assumptions are necessary. Formulae derived from the kinetic theory imply (1) that the reacting molecules have a negligible size, (2) that all the reacting molecules are freely mobile. Neither of these assumptions can be true in the systems under consideration, since the molecules are relatively enormous, and it is practically certain that with the larger viruses many reacting groups are present on, and bound to, the surface of a single virus particle. Langmuir's formula is derived from the single consideration that in a freely reversible system at equilibrium the number of reacting particles reaching the adsorbing surface must equal the number of such particles leaving the surface. In the lack of exact knowledge as to the nature of the bond between antigenic group and antibody molecule, the simpler assumption seems preferable.

If then we have available a method of enumerating the "survivors" obtained with mixtures in which the serum and virus concentrations are varied, we should in the ideal case obtain the following relations:

(1) The proportion of survivors is independent of the absolute concentration of virus present. This is the "percentage law" applied by Andrewes and Elford to bacteriophage neutralization (Chapter III).

(2) Any given system will reach an equilibrium state at a rate proportional to the concentration of antibody and thereafter maintain this equilibrium, i.e. except for very weak sera there will be no progressive change in the number of survivors.

(3) Provided time is allowed to reach an equilibrium condition, dilution of a concentrated serum-virus mixture should give a system of the same activity as one in which the same final concentrations have been reached by mixing the already diluted reagents.

(4) The proportion of survivors will be inversely proportional to the concentration of serum—again, provided time is allowed for mixtures containing weaker concentrations of serum to reach equilibrium, the "C. P = constant" rule.

A graphic representation of these deduced relationships is shown in Figs. 14 and 15.

It will be obvious that our simplifying assumptions are most unlikely to be completely valid for any actual virus-serum reaction. Certain deviations from these assumptions are more likely than others, and we may briefly discuss in what respects these will modify the experimental findings from those to be expected for the "ideal case".

1. Like any other population of living organisms, individual virus particles will differ in the ease with which they are inactivated by physical or chemical agents, including specific antibody. If there is a wide range in susceptibility, a certain proportion of particles will remain viable under conditions which "theoretically" should cause complete inactivation. The result will be that the condition

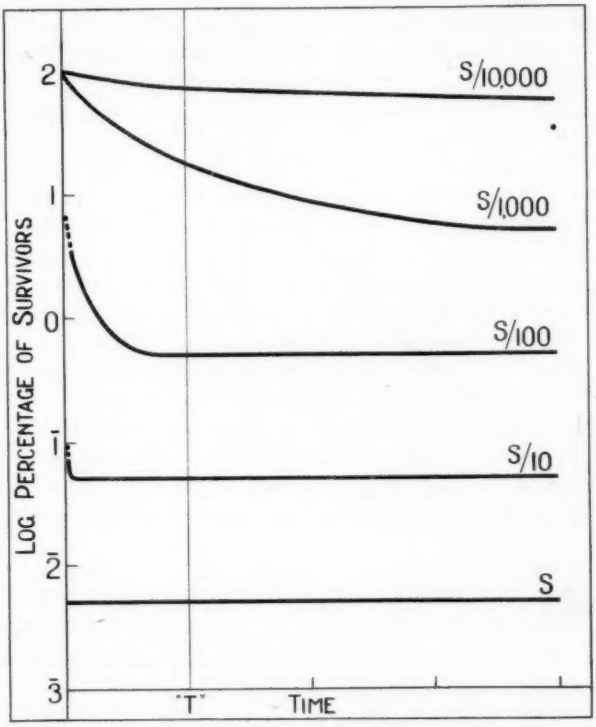

Fig. 14. Schematic presentation of the course of an "ideal" virus-antibody reaction with time.

"C.P = constant" will not hold, and the serum concentration survivor curve will be flattened to some such form as shown in C. (Fig. 15). Any *increased* susceptibility of a similar proportion will hardly be detectable in any type of experiment —it is only the less susceptible particles which influence the results.

2. The time taken to reach equilibrium will be approximately proportional to the concentration of serum. At concentrations below some limiting value, there-

fore, equilibrium will not be reached in any actual experiment where a certain standard time of contact is used. Curve B. (Fig. 15) shows the modification of the serum concentration-survivor curve which will result from the action of this factor.

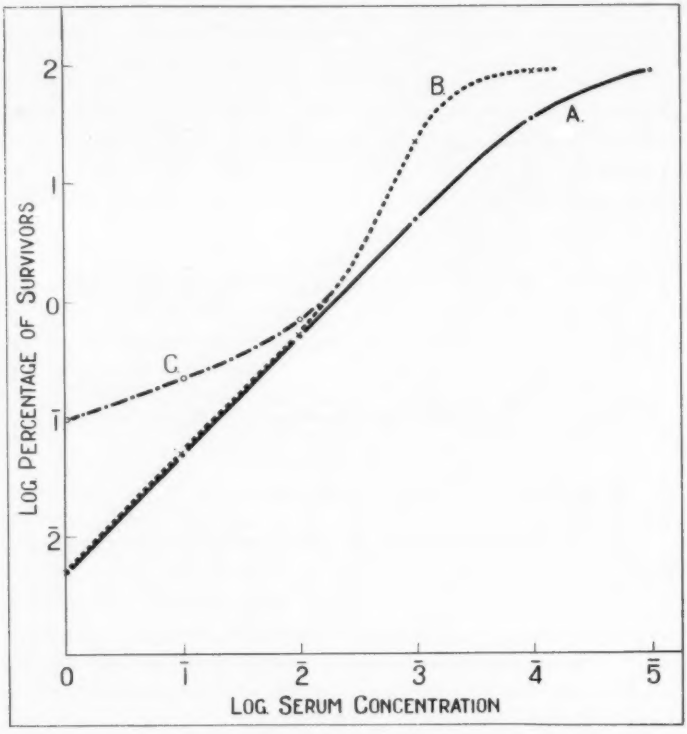

Fig. 15. Theoretical serum concentration-survivor curves.

A. For an "ideal" reaction, in which sufficient time is allowed for all mixtures to attain equilibrium.

B. For an "ideal" reaction, to indicate the results at a standard time after mixing, which is insufficient to allow the more dilute serum mixtures to reach equilibrium.

C. Type of modification introduced if the population of virus particles contains a proportion of resistant individuals.

3. In most of the well-studied immunological reactions it is recognized that antigen-antibody union becomes more permanent with time. There is much evidence that some virus-antiserum reactions at least are not completely reversible by dilution. It is a common finding that when virus-undiluted serum mixtures are diluted, say 1,000 times, and the final diluted mixture compared with a mixture

made by adding 1 : 1,000 virus to 1 : 1,000 serum, the former is non-infective, the latter infective. Examples of such results will be found in several subsequent sections. These irreversible or less readily reversible changes, particularly characteristic of strong serum mixtures, may be of two sorts. In the first place, if the virus is in sufficient amount, aggregation of particles will occur, with corresponding reduction in infectivity, and, as well, the formation of a more stable virus-antibody "lattice structure", which will not readily be broken down by dilution. Such aggregation undoubtedly plays a part in neutralization reactions, but probably not so important a part as is ascribed to it by Merrill (1936). The second mechanism by which irreversibility can develop with low virus concentrations in which aggregation is insignificant, is by the development of firmer union between antibody molecule and the virus surface. The simplest conception of this is that under the influence of molecular bombardment an antibody molecule already held by one bond to the virus surface is brought into such a position that another linkage between an antigenic determinant and corresponding area on the antibody molecule becomes possible. The frequency of this occurrence would be approximately proportional to the amount of antibody already bound. If either of these less freely reversible processes plays an important part, they will be experimentally demonstrable (a) by a progressive fall in the proportion of survivors with time, and (b) by discrepancies in the results of dilution experiments. The effect on serum concentration-survivor curves will in general tend to produce a steeper curve, since there is an unduly great inactivation with concentrated serum. This effect is in the opposite sense from the deviation due to heterogeneity of virus particles, and it is not unlikely that some of the experimental curves to be shown later approach the ideal type largely because these two modifying factors more or less cancel each other.

4. The assumption that the degree of inactivation is proportional to the average amount of antibody bound to the virus particles is merely the simplest *a priori* hypothesis. We have made no attempt so far to discuss the mechanism by which union of antibody to virus diminishes the probability that infection will occur, and we consider that this discussion may be more conveniently deferred until the experimental work has been presented (Chapter VI).

The egg-membrane method of titration is particularly well adapted for the verification or otherwise of these points, since the results are obtained in the form of the average number of focal lesions observed on membranes inoculated with the mixture in question. There is a direct proportionality between the concentration of virus and the number of pocks, and each pock can be taken as representing one survivor in the sense used above.

Full details of the methods used in titrations on the chorioallantois are contained in a recent monograph (Burnet, 1936), and need not be given here. The accuracy of the titrations varies somewhat with different viruses, but with those we have used for detailed studies, the following statements are approximately cor-

rect: If five membranes are used for each mixture, the average count (excluding a small proportion of unsatisfactory membranes) will be within ± 20 p.c. of the true count; with three membranes, the smallest number used in any experiment, ± 50 p.c. Different batches of eggs vary somewhat in the regularity of the results obtained, and at times the counts have been well within these limits. This degree of accuracy is insufficient to allow any positive statement that a given formula represents the course of the reaction, but the method is more accurate than any other practicable method of virus titration, and allows the statement that the results are not incompatible with the consequences of a given hypothesis to carry more weight than it otherwise would.

We are aware that any attempt to express the results of a complex reaction between biological materials in any simple mathematical form is bound to be incomplete, owing to the inevitable complicating factors. Further, it is a valid criticism that in problems like the present one, the accuracy of experimental work is too low to allow proof that any postulated formula does really provide an accurate representation of the facts. As Clark (1933) points out, in dealing with pharmacological problems of somewhat similar type it would be easy to produce several formulae, each corresponding to quite distinct physicochemical mechanisms, but each capable of giving, with appropriate arbitrary constants, curves which would fit experimental results of the present degree of accuracy. Our aim is certainly not to *prove* the accuracy of our hypothesis by the approximate quantitative agreement of the experimental and expected results, but to show that a hypothesis developed from the accepted body of general immunological theory predicts results which are not incompatible with the experimental facts, and hence to show that the immunological reactions of viruses do not require the postulation of any essentially new processes.

2. Aggregation and Complement Fixation Reactions.

In addition to the protective or neutralizing function of antiserum, certain *in vitro* reactions of virus or virus products with immune serum can be observed, and must be considered in relation to the former function. These are the aggregation reactions, agglutination of virus particles or specific precipitation with soluble virus antigen, both chiefly studied with material from vaccinia lesions, and complement fixation reactions. Until very recently, neither of these types of reactions had provided much information of value, but with the development of new technical methods for concentrating virus particles, they are becoming increasingly important.

Merrill (1936) has discussed the conditions under which aggregation reactions with viruses can be expected to occur. As he points out, the most important consideration is the "mass factor", i.e. how much actual virus antigen, having regard both to the number of particles and the size of the particles, is present in the virus preparations available. A rough approximation to the available experimental re-

sults is given by the assumption that 0·001 mg. of antigen per c.c., irrespective of the particle size or molecular weight, is the minimal amount capable of producing a visible aggregation with an active homologous immune serum. Over the whole range of antigenic particles from relatively simple molecules through virus particles to bacteria and red blood cells, there is a consistent deviation from this approximation, the smaller antigens (molecules) reacting with somewhat smaller numbers per c.c., while the larger particles (bacteria and red blood cells) require

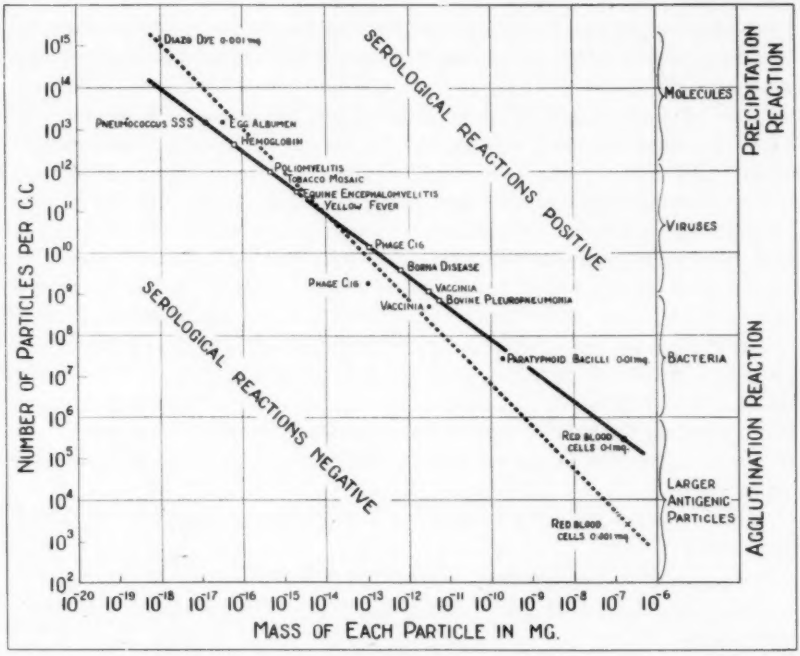

Fig. 16. Merrill's diagram slightly modified to illustrate the significance of number and mass of antigenic particles in determining whether visible aggregation reactions occur. Black dots indicate experimentally determined values: open squares, interpolated values. From Merrill, 1936.

to be in considerably larger numbers. Merrill's table (Fig. 1, p. 175 of his paper) is reproduced (Fig. 16). From this it will be seen that in the case of vaccinia virus the suspension must contain approximately 10^9 particles per c.c. to show agglutination by immune serum. This titre is a good deal higher than that reached by any ordinary emulsion of infected tissue, but can be easily exceeded with Craigie's methods. For the smallest viruses much larger concentrations would be needed, for yellow fever about 10^{11} per c.c., for poliomyelitis about 10^{12}.

In all these calculations it must be recognized that the infective titre of a virus preparation can provide only a minimal value for the number of virus particles present. Only a proportion of viable particles may be capable of inducing infection, and both non-viable particles and soluble antigen derived from the virus may function in aggregation reactions. Because a virus preparation is only infective to a titre of 1 : 1,000, it does not necessarily follow that it is hopeless to look for aggregation reactions. Amies (1937) has obtained particles from Rous sarcoma tissue which carry the infectivity and are specifically agglutinated by immune serum. Their number, however, is far higher than it would be if each particle were capable of inducing infection. The even more striking instance of the low infectivity of the tobacco mosaic virus protein of Stanley has been discussed in the section on plant viruses.

Apart from the aggregation reactions of vaccinia virus and its products, which will be discussed in a later section, work along these lines has been reported for varicella and zoster (using vesicle fluid as source of virus) (Amies, 1934) and for the Rous tumour virus by Ledingham and Gye (1935), Amies (1937).

Complement fixation reactions are somewhat more sensitive than precipitation ones. The accepted theory of the reaction with soluble antigens is that fixation occurs best at the stage when particulation is just commencing, and the aggregates are very small (Dean, 1912, Goldsworthy, 1928, Goodner and Horsfall, 1936). The optimal antigen antibody ratio for complement fixation is regularly lower than for precipitation. Merrill considers that the difference in favour of the complement fixation "would appear to be within a factor of ten times. This means that, on the scale presented, the curve for complement fixation would fall not more than one place on the logarithmic scale below the solid line given for precipitation-agglutination reactions".

Satisfactory complement-fixation reactions have been described for the following viruses:

Psittacosis: Bedson (1935).

Vaccinia: Gordon (1925), Bedson and Bland (1929), Gilmore (1931), and many others.

Herpes: Bedson and Bland (1929).

Varicella and Zoster: Bedson and Bland (1929), Brain (1933).

Influenza: Smith (1936), Fairbrother and Hoyle (1937).

These are all relatively large viruses which can be obtained in considerable concentration in natural lesions.

Dog Distemper: Laidlaw and Dunkin (1931). Size of virus unknown.

Rift Valley Fever: Broom and Findlay (1932).

Yellow Fever: Frobisher (1929).

The last two viruses are of small particle size, 23–35 mμ and 17–28 mμ respectively, and would hardly be expected to react on Merrill's hypothesis. Both viruses, however, are present in high concentrations in infected tissues, as judged by titrations

of infectivity, and since antigen in the form of "dead" non-infective particles or soluble substance is equally available for the complement fixation reaction, the discrepancy may not be significant.

Summary.

In summary, the point of view we shall adopt is as follows: In dilute suspensions of virus particles where the chance of collision between any two is small, the presence of antibody results in the development of a reversible union between antibody molecules and certain determinant groups on the virus surface. When a certain amount of antibody has united, the virus particle becomes non-infective—the amount necessary may differ according to the method by which the infectivity of the particle is tested. If the concentration of virus is high enough, and sufficient time is given, collisions of the partially-coated particles result in the formation of aggregates according to the principle of lattice formation enunciated by Marrack. Such aggregation is essentially identical with bacterial agglutination or specific precipitation of soluble antigen, merely representing the reaction of antigenic particles of intermediate size. It can be detected either by the method of complement fixation or by the observation of macroscopic agglutination.

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Chapter VI.

VACCINIA VIRUS.

1. In vitro Aggregation Reactions.

For technical reasons the study of the *in vitro* aggregation reactions of vaccinia virus is much further advanced than that of any other animal-pathogenic virus. The method of Craigie (1932) has rendered it practicable to obtain large amounts of crude material very rich in the elementary particles of the virus and their soluble derivatives. The virus particles have been shown by several independent methods to be relatively large, about $0{\cdot}15\mu$ in diameter, and there is no difficulty in collecting and washing them in a high speed centrifuge. The virus is conveniently removed from a crude suspension by filtration through a Seitz disc which retains the particles but allows soluble substances of specific reactivity to pass through in the filtrate. Both washed elementary bodies and Seitz filtrates react with immune rabbit serum by agglutination or precipitation. Agglutination of elementary bodies can be observed either microscopically (Ledingham, 1931) or macroscopically (Craigie, 1932) and active study of this reaction in recent years has shown that in no essential respect does it differ from a typical specific bacterial agglutination, e.g. that of the pneumococcus by homologous antiserum.

We shall pass over the important preliminary work which made these technical advances possible (Gordon, 1925, Burgess, Craigie and Tulloch, 1929, and Craigie and Tulloch, 1931) and discuss only the results of recent work, particularly those of Craigie and Wishart and of Parker and Rivers. The crude lapine used for such studies is obtained by means of a uniform heavy inoculation into the scarified skin of a rabbit, the infected tissue being harvested three days after inoculation. The crude emulsion is lightly centrifuged to remove cell fragments, then subjected to high speed centrifugation in an angle centrifuge to throw down the elementary bodies. The supernatant fluid is filtered through a Seitz disc to remove remaining virus particles, and the filtrate retained. This filtrate reacts with immune sera, and the responsible soluble specific substances can be conveniently referred to as S.S.S. Further washing of the virus particles in saline or broth results in the liberation of further amounts of S.S.S. into the supernatant fluids. This indication that S.S.S. is a derivative of the virus particles is supported by the detailed immunological study of the two types of material. It will be convenient to discuss first the reactions and properties of the soluble factors in Seitz filtrates.

Craigie and Wishart (1936a and b) and Wishart and Craigie (1936) have obtained similarly reactive material both in filtrates from crude vaccine pulp and by dissociation from elementary bodies during the process of washing. The S.S.S. precipitates with hyperimmune rabbit serum and by cross absorption tests

(Craigie, 1932) the antibodies responsible are the same as those causing agglutination of washed elementary bodies. Heating the filtrate above 56° C. results in the destruction of portion of the precipitin activity. Suitable absorption tests show that the antigen present possesses both heat labile, L., and heat stable, S., aspects. Hyperimmunization of rabbits having a basal immunity conferred by dermal vaccination some months previously, with unheated (L.S.) and heated (S) specific filtrates showed that both preparations were antigenic, producing L. and S., and S. antibodies respectively. By suitable immunization and absorption procedures, pure L. and pure S. antisera could be obtained. Either serum, however, precipitated the original S.S.S. completely, so that the responsible antigen must be in the form of a complex (L-S) rather than as a mixture of L. and S. components.

Wilson Smith (1932) obtained a heat stable specifically precipitable substance by autolysis of rabbit testis infected with a strain of neurovaccinia. Proteins were precipitated by boiling at an acid reaction and the clear supernatant fluid was used for the experiments. Though stable at 100° C., its activity was destroyed by autoclaving. This material precipitated with immune serum, but apparently much less of the active agent was present than in filtrates prepared by Craigie's methods. The latter author mentions preparations active when diluted 1:1,600, while Smith's material was not precipitated at dilutions beyond 1:16. It is doubtful, therefore, whether Smith's finding that his agent was non-antigenic is justified: there may have been too little antigen to provoke a detectable response.

When washed elementary body suspensions are mixed with immune serum, macroscopic precipitation occurs, producing well-defined floccules. Microscopic examination of the precipitates shows that they consist solely of clumped masses of elementary bodies, i.e. the reaction is a true agglutination. Analysis of the reaction along the lines familiar in bacteriological work shows that the washed elementary bodies possess the same L. and S. antigens as the soluble antigens. Treatment of the virus particles by heat (56° C.), formalin or methylene blue in the presence of light removes, or greatly diminishes, both reactivity to L. antibody and power to induce its formation in inoculated rabbits. The S. agglutination is stable at 95° C., but is somewhat impaired in agglutinability, though not in absorptive capacity at 100° C. There is evidence that infective elementary bodies contain additional antigenic material beyond the L.S. complex. If rabbits are hyperimmunized with infective elementary bodies, and the serum absorbed with L.S. soluble antigen till non-reactive with this, it will still agglutinate elementary bodies to about one-fourth of its original titre.

Complement fixation with vaccinial material and immune serum has been described by many authors, but its relation to the aggregation reactions is not yet clear. Since boiled antigens have been used successfully, the S. antigen of Craigie is presumably capable of fixing complement with the appropriate antibody, but this does not exclude the possibility of the L. antigen also being effective in this respect under suitable conditions.

A skin reaction of allergic type can be elicited in immune animals (rabbit or man) by killed, washed suspensions of elementary bodies, or by virus-free filtrates. Wilson Smith (1932) found that all but one of the human subjects tested who had been vaccinated reacted to his heat stable soluble substance. The accelerated reaction of a human revaccination is probably a manifestation of the same phenomenon. Craigie (1934) found that a similar reaction could be obtained with killed elementary bodies.

The important question of the relationship between protective antibodies and those responsible for these *in vitro* reactions has not yet received the attention it justifies. Although Wilson Smith (1932) presented certain evidence that the "neutralizing" and precipitating antibodies were the same, later data suggest that no simple relation of identity or non-identity is possible. Parker and Rivers (1936) found, for example, that immunization with formolized dead elementary bodies gave a serum in which the ratio: *agglutinating titre/protective titre* was much larger than in serum from a rabbit immunized by infection with living virus. Sera obtained after immunization with virus-free filtrate had a similar character to the former type, but their antibody content was lower. Craigie and Wishart (1936b) note that a serum produced by hyper-immunization with infective virus particles may be absorbed with S.S.S. to remove all antibody reacting with L. and S. components, yet still retain power to agglutinate elementary bodies. It seems highly probable that such an absorbed serum would also retain protective activity, though no data are given on the point.

These aggregation reactions between elementary bodies or virus-free filtrates and anti-vaccinial sera do not involve any consideration of the susceptible cells, and can be discussed in the same terms as one would the immunological reactions of bacteria *in vitro*. The detailed resemblance to bacterial reactions is, in fact, immediately obvious. The heat stable preparations of Wilson Smith are closely analogous to the polysaccharide haptenes which have been isolated from most pathogenic bacteria. It is now certain that these haptenes do not occur as such in the living bacteria, but form part of more complex and labile molecular groupings on the bacterial surface (cf. Heidelberger, Kendall and Scherp, 1936, Boivin *et al.*, 1933, Gough and Burnet, 1934). As yet there is only fragmentary information in regard to the chemical nature of the precipitable substances of vaccinia, but what there is points rather strongly toward a complex polysaccharide being at least partly concerned. In many respects the relation of the L.S. complex of vaccinia filtrates to the S. component of heated material is immunologically equivalent to that between acetyl-polysaccharide and the originally prepared specific soluble substance of the pneumococcus. In each case the more reactive and more complex substance approaches more closely in its reactions the living bacterium or virus. It is clearly impossible, however, to disrupt an organized living surface into "soluble" fragments and retain the whole of its antigenic specificity, and with refined methods some differences will always be detectable in the nature of the

active immunity and the antibody content of the serum resulting from immunization with killed virus or soluble products when these are compared with the changes observed after infection with living virus.

Just as absorption of antipneumococcal serum with S.S.S. left the "protective antibody" only slightly reduced, so it cannot be expected that absorption of antivaccinial serum with any form of modified virus will remove the whole of the protective antibody. The available evidence on the point is decidedly unsatisfactory, owing to the practical impossibility of making accurate antibody titrations by current methods. Perhaps the most significant evidence is that of Parker and Rivers (1936) who showed that immunization with virus-free filtrates could produce a low grade of active immunity and protective antibodies in the serum in rabbits. More definite development of such protective antibodies is recorded by Salaman (1934), who found that the active agent in Seitz filtrates could be greatly increased in antigenicity if it were adsorbed on collodion particles. Salaman's results are consistent, but no mention is made of precautions to prevent spontaneous subclinical infections of his animals by vaccinia. This is a well recognized possible source of error in such experiments, and his results must be accepted with some reserve.

To summarize these studies on *in vitro* immunological reactions of vaccinia virus, they show that the virus particles can most usefully be regarded simply as tiny bacteria. They have an antigenically active surface structure from which fragments of varying degrees of complexity and heat stability can be liberated into solution. The whole range of determinant groups amongst the antigenic constituents of the virus surface will be represented amongst the population of modified globulin molecules which comprise an antiserum, and as in other similar fields, it is easy to separate from this population by appropriate absorptions fractions which react particularly with some partial or modified antigenic component. This practicability of dividing the antiserum into fractions of different reactivity does not necessarily indicate that the corresponding antigen fractions exist as such in the natural antigen—the living surface. From our present point of view, the most important feature is probably the demonstration that typical firm union of antibody and antigenic molecule or particle allowing absorption procedures can occur *when the reagents are sufficiently concentrated.*

2. Neutralization of Vaccinia Virus.

The interest of investigators in the reaction between vaccinia virus and homologous antisera has always been largely centred round the reduction in infectivity of the virus, the so-called neutralization of virus by immune serum. We shall conform to ordinary usage in regard to this terminology, although we feel that the successive transfer of the term "neutralization" from the chemical reaction between acids and alkalies to the toxin-antitoxin reaction and to the virus-antiserum reaction has little justification.

Experiments on such neutralizations all take the form of the inoculation of mixtures of virus and antiserum into the tissues of susceptible animals. The effect of the antiserum is judged by the nature and extent of the lesions which develop in the animal after some convenient arbitrary period, in comparison with those produced in the absence of serum. The species of animal and the particular tissue used for inoculation both play an important part in determining the result of inoculation of serum-virus mixtures, and it will be convenient to discuss the results in sections, each dealing with one of the more commonly used methods.

(A) *Neutralization on the chorioallantoic membrane of the developing chick.*

Although this is the most recent method to be used in the study of the inactivation of vaccinia virus by immune serum, the findings and their interpretation in the light of the general considerations put forward in Chapter IV will be discussed first. In many respects the conditions are simpler than when orthodox animal inoculations are used, and the results are obtained in a direct numerical form which allows easy construction of time-survivor and serum concentration-survivor curves.

When suitably diluted suspensions of vaccinia virus are inoculated on egg membranes, discrete readily-counted pocks appear on the membranes, each pock representing a viable vaccinia particle. When serum-virus mixtures are inoculated, the number of lesions appearing is reduced in comparison to controls. (Keogh, 1936). Such lesions show no detectable differences in size and appearance to the controls; there appears to be a quantitative reduction in the number of virus particles capable of initiating lesions, without any evidence of qualitative change. The dermotropic and neurotropic strains which we have studied produce recognizably different lesions, the former being of a proliferative type, the latter more necrotic and markedly haemorrhagic. It might have been expected that pocks arising from serum-neurotropic virus mixtures would tend to show the characteristics of the less virulent dermotropic lesions, but this does not occur.

(i) *The Percentage Law.* Within the experimental range, mixtures of vaccinia virus with immune serum show a percentage reduction depending on the serum concentration, but independent of the virus concentration. Since in all such experiments dilution of the initial serum virus mixtures is inadmissible, only a limited range of virus concentrations is available for testing this point. We have observed no indirect indications that it fails to hold over the whole experimental range, and in calculating results with vaccinia and all other viruses tested in this way, it is assumed that the "percentage law" does in fact hold for all concentrations of virus and serum used.

(ii) *Progress of inactivation with time.* A number of experiments failed to show any "time effect", the counts from mixtures of virus and serum tested immediately after mixing being the same as those obtained when the mixtures had stood two hours before inoculation into eggs. With a suitable serum, a definite

time effect can be shown by either of two methods. If virus and antiserum are mixed rapidly, shaken, and standard volumes of the mixture inoculated within a few seconds on previously prepared eggs, the counts are higher than those obtained when the same mixture is inoculated after standing for a few minutes. There is a rapid attainment of an equilibrium level, which, under the usual experimental conditions, remains almost constant from five to ten minutes onward.

The second method of demonstrating a "time effect" is by mixing concentrated virus and serum and (a) immediately after mixing, (b) after a time interval diluting the mixture beyond the effective concentration of serum (e.g. 1 : 10,000). Here also the counts are lower in the dilution from the mixture in which serum and virus have been in contact longer. This method allows a simpler demonstration of the time effect than the first method. The conditions involved in these different methods of demonstrating the importance of time of contact between virus and antiserum are discussed later in Chapter VIII. Here it need only be noted that both types of experiment provide direct evidence of virus-antibody union being concerned in the inactivation. Further evidence in the same direction is provided by the fact that if mixtures containing the same amounts of virus and serum are prepared by (a) dilution of a mixture of both components, (b) separate dilution of each component and subsequent mixture, fewer lesions arise on the membrane inoculated from the mixtures in which virus has had initial contact with concentrated serum. As in all experiments of this type, the observed result may depend in variable proportion on (a) secondary "irreversible" union of virus and antibody in the concentrated mixture, (b) aggregation in the concentrated mixture, (c) insufficient time being allowed for the initially dilute mixture to reach equilibrium.

(iii) *The serum concentration-survivor curve.* Much of the data to be presented in regard to vaccinia and other viruses which are susceptible to chorioallantoic membrane titrations, will be in the form of serum concentration-survivor curves. The expression "percentage of survivors" is used throughout to express the percentage relation between the number of pocks which would have been produced from the same amount of untreated virus on the assumption that there is a linear relation between the number of pocks produced and the concentration of virus. "Survivor" thus does not mean a potentially viable virus particle, but one which actually does produce a visible lesion.

TABLE 8.

Relation Between Serum Concentration and Percentage "Survivors" for Vaccinia.

Serum Final Dilution.	Titration. Expt. 1.	Expt. 2.	Expt. 3.	Observed Average.	Calculated. A.	B.
1 : 2	1·4	1·6	1·1	1·4	0·20	1·4
1 : 20	3·6	5·0	3·2	3·9	1·96	3·2
1 : 200	19·0	17·0	16·0	17·0	16·7	17·9
1 : 2,000	60·0	33·0	47·0	47·0	67·0	68·0

See text for method of calculating A. and B.

In presenting the results of such experiments graphically, the only practicable method is to use a logarithmic scale for both abscissae and ordinates, as was done in Fig. 15, which shows the serum concentration-survivor curves to be expected from certain ideal cases. The experimental error implicit in the method is represented by similar magnitudes at all points on such curves, instead of being greatly magnified when there are more than 20 p.c. of survivors, as it is in a curve using arithmetical co-ordinates. Details of the action of weak serum in reducing the count from 100 to 90 p.c. might be of great interest from the point of view of

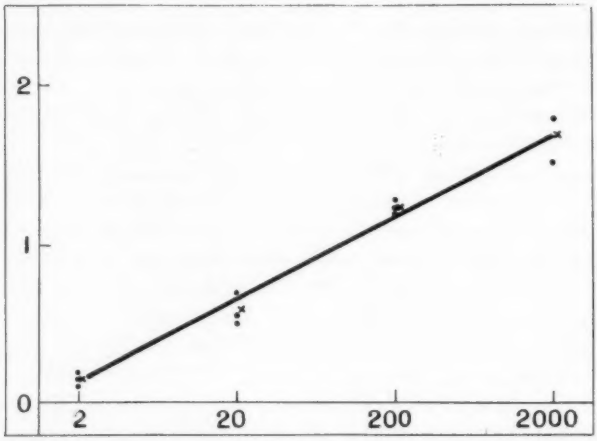

Fig. 17. Serum concentration-survivor curve of vaccinia virus and antiserum. Dots indicate the values obtained in three different experiments, x the average value. Log. percentage survivors plotted against final serum dilution.

determining whether the range of variation in susceptibility was of a normal form, including some individuals more susceptible as well as some less susceptible than the average, but there is no possibility of obtaining them with a technique of this sort. Table 8 gives a series of results obtained with an active serum. If we calculate the number of survivors from the formula $C \cdot \frac{P}{100 - P} = K$, taking K as 1,000, the results in column A. show that the more concentrated serum is less active than is to be expected, while the most dilute serum is more active. Fig. 17 shows the corresponding serum concentration-survivor curve, which may be compared with curve C. of Fig. 15. A simple explanation of this discrepancy is that the virus particles differ widely in the ease with which they can be inactivated by serum. If we assume that with this serum 98·8 p.c. inactivation only is possible, the remaining 1·2 p.c. being insusceptible to any concentration, the calculated results become those in column B., which show a closer agreement to the facts.

With weaker convalescent sera there is a still further deviation from the "C.P. = constant" rule. Although an approximation to a straight line is obtained when the logarithm of the serum concentration is plotted against the logarithm of the concentration of surviving particles, the slope is much flatter. The data can be fitted by a formula of the type $kC^{\frac{1}{n}} = \frac{100 - y}{y}$ where $n > 1$. With these weaker sera, it is difficult to get results of much value, as the range of serum dilutions which can be used is limited, and the differences between the numbers of survivors in mixtures containing successive tenfold dilutions of serum are often small and within the range of error. Results of this nature are explicable on the assumption that adsorption of antibody occurs according to the Freudlich isotherm. But the fact that the more active the serum the more closely the value of n approaches unity, suggests that the true explanation is to be sought in the heterogeneity of antibody molecule and virus particle populations.

We have already discussed similar problems of varying susceptibility in regard to bacteriophages in Chapter III, and have elaborated the conception of varying avidity of antigen-antibody union in this connection. The wide range of variability in a population of living organisms is always in evidence in any studies of rate of inactivation by heat, disinfectants, etc., as well as by immune sera. Further, the apparent extent of the range of variability will be greater with qualitatively less active agents, e.g. heat at 55° C. compared with 100° C. If the weaker convalescent sera contain a large proportion of low grade less avid antibody compared with the more avid antibody of hyperimmune serum, then the flat type of survivor curve would be expected, since it is merely an expression of the wide range in susceptibility of virus particles to inactivation by such antibody. On this view, a hyperimmune and a convalescent serum might both be capable of effecting 50 p.c. inactivation at a dilution of 1 : 1,000, yet the hyperimmune serum would be relatively much more efficient when used undiluted, owing to its greater power to combine with and inactivate the small proportion of virus particles intrinsically resistant to the action of immune serum.

(B) *Neutralization of vaccinia in the rabbit.*

(i) *Tests by intradermal inoculation.* Most recent workers on vaccinal immunity have preferred to use intradermal injections as the method of titrations, Levaditi's neurovaccine being generally used as the virus strain. Earlier workers used scarification methods and dermal strains, usually of calf lymph. Their results will not be discussed in detail. By the intradermal technique, closely reproducible results can be obtained in direct titrations of virus by tenfold dilutions. When the method is applied to testing the activity of immune sera, however, wide discrepancies between the results obtained on different rabbits have been observed, particularly by Andrewes (1928, 1929). In one example given by Andrewes, seven successive titrations of a serum on different rabbits appeared to indicate that 200, 20, 200,000, 20, <20, <20, 200,000 minimal infective doses were neutralized by

1 c.c. of serum. No significant differences were noted in the susceptibility of the same seven rabbits when concurrent direct titrations of virus diluted in Ringer solution were made. Similar irregularities have been recorded by other workers, e.g. Parker and Rivers (1936), though the range of variability recorded has been less than that obtained by Andrewes.

This irregularity of results makes it almost impossible to provide any quantitative data in regard to the relationships between serum and virus concentrations and the result of inoculation. In limited tests, using one animal only for all the mixtures being compared, Andrewes (1928) found no proportional relationship, the undiluted serum almost always neutralizing more than ten times as much virus as serum diluted 1 : 10, while serum diluted 1 : 100 rarely showed any neutralization at all.

(ii) *The time factor in protection and neutralization experiments.* Andrewes (1928), Long and Olitsky (1930) and Sabin (1935a) are all agreed that mixtures made and inoculated immediately show the same degree of neutralization as mixtures allowed to stand some hours at 37° C. Andrewes also found that the phenomenon of "reactivation by dilution" took place equally well whether the undiluted reagents had been in contact for a few minutes or for 24 hours. With mixtures held in contact for longer periods (two to four days at room temperature) Andrewes (1930) found that a progressive loss of ability to be reactivated by dilution took place. Recovery of virus by precipitation with euglobulin, however, was more successful, mixtures held four days at room temperature being almost as readily dissociated in this way as freshly prepared mixtures. Finally, Sabin (1935a) finds no greater difficulty in recovering virus by centrifugation from mixtures kept seven days than from recently made mixtures. His protocol, however, indicates a 90 per cent. inactivation when the time of contact was prolonged to fourteen days in the refrigerator.

It seems clear that an initially completely reversible union is slowly converted to one of progressively firmer character. It must be recognized that the sera used by different workers may well have differed considerably in the rate at which they induced irreversible inactivation. If we allow for this, and also for the variability of result in inoculating serum-virus mixtures, there seems to be no serious incompatibility in regard to facts amongst the authors who have studied this problem. Also, since there is nothing in Sabin's experiments to allow differentiation between a freely reversible union and no union at all, the former interpretation is not opposed to his general point of view.

An experimental fact of importance for understanding the conditions of neutralization in the rabbit skin is Andrewes' demonstration of the effects of inoculating virus and serum in the same spot at different intervals. If an adequate dose of serum is inoculated either simultaneously with, or at any time up to 24 hours before, the virus, no lesion results. If serum is given five minutes later than the virus, infection invariably occurs, though the resulting lesion is naturally smaller than the control without serum.

(iii) *Absorption of protective antibody.* As Andrewes (1928) showed, an "underneutralized" mixture of vaccinia virus and immune serum contains a large excess of antibody. Removal of the virus by passage through a suitable filter gives a non-infective filtrate whose neutralizing power is not detectably lowered. Sabin (1935a) confirmed this. Under the circumstances, one could only expect absorption of protective antibody to be demonstrable with an enormous excess of virus. Wilson Smith's (1930) experiments with vaccinia and herpes serum showed a definite small degree of specific absorption when large amounts of infected testis were used to absorb. The most satisfactory way to determine whether such absorption occurs would clearly be to use large amounts of elementary bodies. Craigie (1932) has shown that agglutinating and flocculating antibodies can be absorbed from immune serum by vaccinia particles, but so far as we are aware, no account has been published of the effect of such absorptions on the content of protective antibody.[1]

(iv) *Neutralization as tested by other routes in the rabbit.* Intracerebral inoculation has been used as the method of testing infectivity by a few authors. Andrewes (1928) found little indication that any inactivating effect was produced at all in such tests. Fairbrother (1932) reports one experiment in which a certain mixture of virus and immune serum was inoculated intracerebrally after varying periods of contact at room temperature. Inoculated immediately after mixing, all three rabbits died in five days; after one hour's contact, death occurred on the sixth and eighth days; after four hours, all rabbits inoculated survived, though three showed definite symptoms of encephalitis. In a subsequent paper (1933), though protection against small doses of virus could be regularly accomplished, Fairbrother found no such influence of time of contact on the result. Sabin (1935d) considers that infection occurred in practically all Fairbrother's experiments, and is inclined to regard the attenuation of the infection as of little significance. His own experiments, however, show that, with a large dose of serum, a certainly lethal dose can be converted into a non-lethal, though still infective, one.

Intravenous inoculation of test mixtures seemed to Andrewes to provide on the whole more uniform results than intradermal tests. The degree of protection (judged by the absence of generalization of the infection) was very inferior to the "best" results obtained by the intradermal method, but by no means insignificant. There is a good deal of evidence that phagocytosis by circulating leucocytes may play an important part in determining the result of such intravenous inoculations (Wilson Smith, 1929, Fairbrother, 1933).

It is probable that the conditions in inoculations by these two routes approach more closely to those of chorioallantoic membrane inoculations than do intradermal tests. The significance of such differences in apparent infectivity according to the method used for test inoculation will be discussed in the following section.

[1] Salaman (Brit. J. exp. Path., 18, p. 245, 1937) has recently shown that protective antibody can be removed by absorption with a suitable dose of washed elementary bodies.

3. The Influence of the Method of Titration on the Results of Inoculating Virus-serum Mixtures.

A given mixture of vaccine virus and antiserum may appear quite non-infective when inoculated intradermally in the rabbit, produce foci corresponding to 0·1 p.c. of survivors on the egg membrane, and appear to be fully infective when inoculated intracerebrally in the rabbit. This difference in result according to the route of inoculation used has been observed with almost all the viruses which have been studied in this fashion. It provides one of the main difficulties in accepting the view that virus-antibody union *in vitro* is responsible for the observed inactivation, and calls for a discussion in some detail of the mechanism by which union of antibody to the virus particle prevents, or fails to prevent, infection.

The conditions on the egg membrane are simpler than those associated with the use of the rabbit skin as a titration method, and we may first consider the mechanism by which union of antibody to virus particles may be expected to reduce the number of foci appearing on the susceptible membrane.

In general, this problem must be attacked in much the same way as was adopted for bacteriophages, but the conditions are more complicated, and the details of the reaction between virus and susceptible cell are much less clearly understood than those of the phage-bacterium interaction. We may summarize some of the relevant considerations as follows:

1. Each vaccinia particle must carry antigenic determinants capable of uniting with a large number of antibody molecules. Consideration of the size of the average vaccinia particle and the average globulin molecule will show that if the entire surface of the particle were covered by a unimolecular layer it would accommodate roughly 10,000 antibody globulin molecules.

2. It is probable that active surface groups, analogous to the B. groups of bacteriophages, exist whose function is to come into specific relationship with components of the susceptible cell. Blocking of these groups by antibody might have a specially significant effect in reducing infectivity.

3. Any virus suspension will be a population of particles with varying degrees of resistance to antibody. Variable resistance to any inactivating agency is so universal a characteristic of biological populations that it is hardly necessary to consider the details of individual variation which might be responsible. Similarly, in any antiserum the antibody consists of a heterogeneous population of modified globulin molecules of varying degrees of reactivity.

4. Results of egg membrane titrations of virus serum mixtures are as reproducible as those of direct virus titrations. Having regard to the high degree of reversibility of the reaction, this must indicate that initiation of the indicator reaction (development of pocks) takes place within a uniform, and probably short, period of time after inoculation, so that irregular degrees of dissociation by dilution with the embryonic fluids do not occur. The recent results of Perdrau and

Todd (1936) show that 10–14 hours' contact with susceptible cells is necessary before the vaccinia virus particle is protected against the action of methylene blue. A more rapid process than this must be postulated in regard to the initiation of infection, and Andrewes' (1928) experiments on the failure of serum to prevent local infection by vaccinia virus given only five minutes before provide experimental justification for this assumption. It seems reasonable to assume that the virus particles rapidly make permanent contact with the susceptible cells, the rate at which they are thus adsorbed being proportional to the concentration of free particles, i.e. greatest immediately after inoculation. This fixation on the surface of the susceptible cell, though incapable of protecting the virus particle against photodynamic inactivation, in some way stabilizes the particles in the infective or non-infective state determined by the concentration of antibody to which they were exposed *in vitro*.

5. In general, coating of a particle with antibody facilitates its entry or ingestion into phagocytic cells, and it is unlikely that such coating of a virus particle in any way hinders its entry into the susceptible embryonic cells. Once adsorbed to the cell, it is conceivable that a rigid film of protein immediately forms around the particle, preventing dissociation of the attached antibody.

As a working hypothesis to account for the reduction in the number of pocks produced by immune serum, we may suggest the following: When a virus particle partially coated with antibody enters a susceptible cell, this antibody coating is stabilized and the fate of the particle depends on whether a large enough number of active surface groups remain unblocked by antibody to develop the intimate relationship to the cell constituents which is necessary for the particle to grow and multiply. Making allowance for a characteristic range of variation in the ease with which virus particles are inactivated by serum, such a mechanism would account for the rough proportionality between the amount of antibody adsorbed and the degree of inactivation.

This hypothesis provides a useful method of visualizing the conditions, but makes certain arbitrary assumptions about the relationship between virus and cell which are neither established nor, at the present time, susceptible to experimental test.

Further, the assumption that the momentary state of the virus-antibody system at the time of fixation to the susceptible cell wholly determines whether infection of the cell does or does not take place, is questionable. It may be a close approximation to the truth when we have a uniform sheet of susceptible cells like the ectodermal layer of the chorioallantois, but it can hardly apply to all types of susceptible cell, irrespective of tissue or species.

It is probably better to attempt to express the position in as general terms as possible, and to avoid any attempt to fill in such details. In our general discussion of the neutralization reaction we have assumed that *in vitro*, i.e. before contact with susceptible cells takes place, the state of any given virus particle is only

statistically determinable, the chance that it will carry any particular amount of antibody being determined primarily by the concentration of free antibody. Instead of assuming, as we have done above, that once contact with the cell has been made the fate of the virus particle is now individually determined by the particular configuration of the virus-antibody system, an alternative and more logical point of view is to extend a purely statistical determinism to the fate of the particle within the cell, as well as *in vitro*. The nature of the triple interaction of virus, antibody and cell is quite unknown; it is undoubtedly complex. We can best avoid giving an impression of unwarranted intimacy with such reactions by expressing the conditions somewhat as follows:

Contact between a virus suspension and a given concentration of serum *in vitro* results in reversible union of antibody to virus particles with a characteristic distribution of the numbers of particles carrying more or less than the average amount. The average is determined by the concentration of free antibody. When a population of virus particles, each carrying an equal amount of dissociable antibody, becomes attached to susceptible cells, the proportion of primary infections produced will be determined (a) by the amount of antibody carried, (b) by the type of cell exposed to infection.

On this point of view, differences in the responses of different tissues to the inoculation of serum-virus mixtures may be due:

(a) To varying degrees of dilution before contact is made with susceptible cells, so modifying the average antibody coating of virus particles.

(b) To differences in the power of the susceptible cell to dispose of antibody-coated virus particles. On general grounds, one would expect tissues exposed to the external environment, e.g. skin and nasal mucosa, to be more effective in this respect than a sheltered tissue like the brain or the chorioallantois.

(c) A third group of factors is not concerned with the *primary* infection by antibody-treated virus particles, but with subsequent interference with the development of a visible lesion by (i) retention of serum at the site of inoculation, or (ii) rapid active production of antibody. These factors are discussed below, in reference to the special case of intradermal tests in the rabbit.

4. Special Factors Involved in Intradermal Titrations.

Compared with the egg membrane method, the use of the rabbit skin for titration of serum-vaccine virus mixtures is characterized particularly (a) by the high degree of variability in results, (b) by the much higher apparent neutralizing power of undiluted immune serum. The other aspects of the virus-antibody reaction which have been discussed in terms of the egg membrane results can also be recognized by intradermal titration methods. Direct evidence that protective

antibody does act *in vitro* on the virus particle, obtained by experiments using intradermal titration methods, may be summarized as follows:

1. If mixtures containing the same amounts of serum and virus are prepared by (a) dilution from a mixture of undiluted serum and virus, (b) preliminary separate dilution of the two components and subsequent mixture, and the two series of dilutions are titrated in the same rabbit, greater inactivation is exhibited in the mixtures in which the virus has been exposed to concentrated serum and subsequently diluted (Goyal, 1935).
2. "Reactivation by dilution" becomes increasingly difficult to demonstrate with lengthened contact of serum and antibody (Craigie and Tulloch, 1931, Andrewes, 1930).
3. Absorption of protective substance by vaccinia virus has been demonstrated (Wilson Smith, 1930).

Assuming that inactivation of the virus particle is primarily due to fixation by it of antibody, explanation of the wide variation in skin titrations will most naturally be looked for in those factors, present in the rabbit skin, but not on the egg membrane, which might be capable of modifying the result. The most important factors of this sort are probably (1) the retention of antibody inoculated as part of the mixtures at the site of inoculation for some days, and (2) the possibility of very early active production of antibody influencing the development of the lesions.

Before describing the possible mechanism of "neutralization of virus", which is in this context synonymous with "suppression of a macroscopic skin lesion", it is necessary to discuss the manner in which such a lesion develops in the absence of immune serum.

It is assumed that a skin lesion can arise following the infection of a single cell, wherein a virus particle multiplies, and which it kills, liberating descendant particles which in turn infect adjacent cells, the process continuing until the animal's protective mechanism arrests it. The macroscopic lesion is regarded as the inflammatory reaction to the products of cell necrosis. Lateral spread of liberated particles is unlikely to be extensive from anatomical considerations, and the size of the lesion will depend largely on the area of distribution of introduced virus particles. This area again will depend on the number of particles introduced, as they will be filtered off by the tissues during the peripheral spread of the introduced fluid.[2]

[2] It may be noted at this stage that it is not legitimate to assume that all the introduced particles are instrumental in producing the skin lesion. McMaster and Kidd (1936) have shown that all intracutaneous inoculations are in reality intralymphatic, and that if large doses of virus are injected intracutaneously in the rabbit ear, virus rapidly appears in the cervical lymph glands. The expression "neutralization of 10^6 M.I.D. in the skin" assumes that the whole 1,000,000 doses are retained in the skin, whereas in fact it is quite uncertain what proportions of such doses reach the lymph glands, and how much of the dose is retained, and therefore "neutralized" in the skin.

In this conception, the single lesion, which is noted when the vaccinia virus is inserted intradermally, is in reality composite and formed by coalescence of microscopic lesions. This is confirmed by the experiments of Duran-Reynals (1935), who has shown that if dilute vaccinia suspensions are more widely dispersed by the use of testicular extracts containing his "spreading factor", small discrete lesions appear instead of the single lesions of the controls.

Turning to the mechanism by which immune serum modifies or suppresses the lesion, the conditions immediately following inoculation are the same as we have postulated on the egg membrane. The virus particles enter, or become adsorbed to the surface of, susceptible cells, their immediate fate being determined by the amount and spatial distribution of antibody on their surfaces. It is reasonable to expect much the same proportion of particles to initiate primary cell lesions in the skin as to produce pocks on the egg membrane, i.e. with most convalescent rabbit sera from 1 p.c. to 10 p.c. This primary 90–99 p.c. reduction represents *in vitro* neutralization which is independent of the individual.

As an indicator of the infectivity of serum-virus mixtures, the rabbit skin differs in two important ways from the chorioallantois. In the first place, antibody inoculated intradermally remains localized for some considerable time. Andrewes (1928) showed that serum given intradermally 24 hours before the virus protected the local region, but failed to protect if given 72 hours previously. It has already been pointed out that, even in mixtures containing unneutralized particles, antibody is present in huge excess. Such antibody then will be retained at the site of inoculation for many hours, and may modify or suppress the development of an "initiated" lesion, perhaps in some such way as follows:

The virus particles initially capable of infecting enter susceptible cells, multiply and cause necrosis and disintegration of the affected cells. Descendant virus particles are present in large number in such disintegrated cells, but they are subject to the influence of the retained antiserum in two ways. Some will be "neutralized" in the ordinary fashion, but, in addition, partial antibody coating of virus particles under these conditions will greatly diminish the diffusibility of the particles through the tissue, and further hinder the development of a macroscopic lesion. In these two ways the activity of the virus may be brought to a stop, even though initial infection occurred. The lesion is aborted, and is regarded by the observer as negative, since the death of a few scattered cells will not give rise to a readable lesion. Even if conditions are such that a proportion of the first generation of liberated virus particles is capable of infecting adjacent cells, the number of such cells infected is far less than would be the case in the absence of serum. The rate of development of the lesion is greatly retarded, and at each generation the chance of arrest of the process recurs.

Secondly, the rabbit, unlike the chick embryo, is capable of rapid active immunization. Wilson Smith (1929) found that some degree of skin immunity was

present 48 hours after primary inoculation with vaccinia. The general practice is to carry out multiple titrations on the one rabbit, including a control series of virus dilutions; so that during this delayed multiplication of virus, active immunity is developing in the animal, and as the introduced serum is absorbed, the loss may be restored by the animal's own antibody.

In general terms, we have divided the neutralization process in the rabbit skin (or in any other animal tissue) into two parts: (i) the *in vitro* reaction, virus-antibody union, which is independent of the nature of the titration method adopted, and (ii) *in vivo* neutralization by serum retained at the site of inoculation, and in some instances by early actively produced antibody. A third factor may need to be considered when the virus-serum mixture is given by such a route, e.g. intravenously, that considerable dilution is possible before the coated antibody particles can reach susceptible cells. The results will then be equivalent to those obtained in another situation when the same degree of dilution had been made *in vitro*.

Such general conceptions applied to the special case of intradermal tests in the rabbit provide an adequate explanation of the higher activity of undiluted serum and of the wide variation in results from animal to animal. The common observation that lesions in the presence of immune serum are frequently considerably modified, tending to appear later and to consist of scattered pocks instead of a single well-defined lesion (Sabin, 1935a), also falls naturally into line with these ideas. The development of a lesion is dependent on the capacity of the particular skin to retain serum, the capacity of a given animal to respond to an antigenic stimulus, and to the strength of such stimulus (e.g. whether or not multiple titrations are made in the same animal), all factors obviously subject to large individual variations.

It should be noted that reactivation by dilution of a non-infective mixture will theoretically not be demonstrable by the chorioallantoic technique, since by the "C.P = constant" rule a standard volume of any reasonable dilution from a concentrated mixture will give the same number of foci (survivors) as the original. It is primarily because of the disproportionately greater efficiency of strong than of dilute antiserum for the secondary *in vivo* neutralization that the classical reactivation by dilution phenomenon is so readily demonstrable in the rabbit skin. When *dilute* serum-virus mixtures are studied, there is a much closer correspondence between the results of egg membrane and rabbit skin titrations (Table 8A).

From this viewpoint, most of the methods which have been used to demonstrate the reversibility of the union between vaccinia virus and antibody, or to show that active virus can be recovered from serum-virus mixtures, including simple dilution, cataphoresis, inclusion of the virus in clots, centrifugation of the virus (Andrewes, 1928, Sabin, 1935a) have owed their success to the partial or complete elimination of the excess antibody, with consequent suppression or diminution of

the *in vivo* or passive effect, and are correspondingly more successful with more complete removal of excess antibody. At the same time, removal of excess antibody will allow the dissociation of reversibly bound antibody and a corresponding reduction in the degree of *in vitro* inactivation.

TABLE 8A.

Neutralization of Vaccinia Virus in the Rabbit Skin with Graded Dilutions of Antiserum.

Virus dilution:	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Serum dilution:	Rabbit 1						Rabbit 2					
IS 1:10	++	±	−	−	−	−	++	+	−	−	−	−
IS 1:50	+++	+	±	−	−	−	+++	+	+	−	−	−
IS 1:100	++++	++	+	−	−	−	+++	+	+	+	−	−
IS 1:250	++++	+++	++	+	−	−	++++	++	(±)	+	−	−
IS 1:500	++++	+++	++	+	±	−	++++	++	(?)	+	±	−
Control (NRS)	++++	+++	+++	++	±	−	++++	++	(+)	+	+	−

Readings on 3rd day after inoculation.

Readings in brackets on second rabbit were in a loose fold of skin and were poorly developed.

The irreversible secondary inactivation *in vitro* will rarely amount to more than 90 p.c., and is only just detectable by titration by falling dilutions of ten in the rabbit skin. It is so small compared with the apparent neutralization of 10^4—10^6 M.I.D. usually shown when undiluted serum is included in the mixtures, that it has frequently been overlooked. That the observed effect is in reality composite can be demonstrated by consideration of the phenomenon of reactivation by dilution. Table 9 is a protocol taken from Burgess, Craigie and Tulloch (1931). The arrangement of the data is slightly altered and condensed. All titrations were made on the one rabbit, and only the final readings are quoted.

TABLE 9.

To Show Influence of Time of Contact on Results of Intradermal Tests with Diluted Virus Serum Mixtures (Craigie and Tulloch).

Dilutions of mixtures.	Immune serum and vaccinia virus mixed and at once diluted and inoculated.	Immune serum and vaccinia virus 4 hrs. at 37°C. then diluted and inoculated.	Normal serum and vaccinia virus mixed and at once diluted and inoculated.	Normal serum and vaccinia virus 4 hrs. at 37°C. then diluted and inoculated.
10^0	−	−	++++	++++
10^{-1}	−	−	++++	++++
10^{-2}	+	−	++++	++++
10^{-3}	++++	−	++++	++++
10^{-4}	−	−	+++	+++
10^{-5}	−	−	+	+

Modified from Table LXXV, page 97, Craigie and Tulloch, 1931.

Three points are to be noted in this experiment: (1) an apparently inactive mixture becomes active on dilution, but (2) the reactivation is incomplete, and (3) is no longer demonstrable on prolongation of contact of virus and immune serum. In our interpretation, the *in vivo* effect is negligible when the serum is diluted 1 : 100, about 90 p.c. of the virus is inactivated within a few seconds of mixing, and by four hours at 37° C. about 99·9 p.c. has been inactivated. As the inactivation *in vitro* progresses with prolonged incubation, the *in vitro* and *in vivo* zones overlap, and the composite nature of the effect is obscured. Andrewes (1928) noted that reactivation by dilution was not obtainable with all sera, and that the dilution point at which reactivation occurred varied greatly from animal to animal. Since observation of the effect depends on the separation of the two zones of *in vivo* and *in vitro* neutralization, and since the *in vivo* element is, as already shown, peculiarly subject to individual variation, such inconstant results are not surprising.

Evidence supporting this approach is supplied by the further experiments of Craigie and Tulloch (1931) with disinfectants. In such cases, there is no reasonable doubt that the action of the drug is directed against the virus particle. Mixtures of vaccinia virus with quinine hydrochloride (0·025 to 0·25 p.c.) or acriflavine (0·01 p.c.) exhibited a well-marked dilution phenomenon, and it was shown that acriflavine inoculated into the skin before the administration of virus modified or abolished the development of a lesion.

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Chapter VII.

INFLUENZA VIRUS.

Introduction.

In their first publication, Smith, Andrewes and Laidlaw (1933) stated that ferrets which had recovered from the experimental disease were immune to subsequent infection with the virus, and that their sera were capable of inactivating the virus *in vitro*. More detailed study of the immunological reactions became possible only when it had been shown that mice could be infected intranasally from ferret passage virus (Andrewes, Laidlaw and Smith, 1934, Francis, 1934). The Hampstead workers described a standardized technique for immunological tests in mice. They prepared a gradocol membrane filtrate from infected mouse lungs, mixed this filtrate with serial dilutions of immune serum, and after allowing the mixtures to stand ten minutes at room temperature, inoculated them intranasally in mice under anaesthesia. Filtration of the virus is not absolutely necessary, and both Francis and Magill (1935) and Burnet (unpublished) have obtained satisfactory results in neutralization tests when supernatant fluid from ground-up infected lungs was used.

The most striking feature of the immunological results obtained by the Hampstead investigators was the high activity of the immune sera from recovered ferrets or artificially immunized horses. It is unusual to find that a serum from an animal convalescent from a virus infection can inactivate an equal volume of stock virus prepared in the usual way as the supernatant fluid from a 5 or 10 p.c. suspension of infected tissue. Sera from ferrets taken a week or two after recovery from experimental influenza, however, can nearly always be diluted 1 : 100 and still neutralize undiluted mouse or egg virus completely. Most of the immunological work with influenza virus has been concerned with the immediately important problem of the distribution of antibody in human sera, and no detailed report of the theoretical aspects of the reaction as observed in mice has yet appeared.

1. Results Obtained Using the Chorioallantoic Membrane for Influenza Virus Titration.

Influenza virus is in many respects particularly suitable for immunological studies on the chorioallantois, provided a well adapted strain such as that developed by Burnet (1935) is available. This strain, derived from a mild, but widespread, epidemic in Melbourne during the winter of 1935, appears to be quite similar to European and American strains in its pathogenicity for laboratory animals, and is neutralized by a serum I.H.2 prepared against the strain W.S. of the Hampstead workers. The development of pathogenicity for the egg has been

described in detail (Burnet, 1936a), and here we shall give only a brief account of the activity of the adapted strain.

The egg passage strain is highly pathogenic for the embryo, inoculation of relatively large amounts causing death of the embryo in 40 to 48 hours; with minimal infective amounts, death may be delayed until the third or fourth day. The virus is present in the circulating blood from an early stage, and its lethal effect on the embryo seems mainly to result from damage to the capillary endothelium, the most characteristic macroscopic lesion being a massive infiltration of the brain substance with blood from numerous capillary haemorrhages. If eggs are inoculated with 0·05 c.c. of a 1 : 1,000 dilution of the supernatant fluid from a finely-ground emulsion of a heavily infected membrane in 5 c.c. broth and opened 40–48 hours later, discrete foci will be found. Their numbers almost invariably lie between 5 and 30. About half the membranes will show easily counted discrete foci; on the others secondary foci will be observed which often make counting difficult. Secondary foci are those which result from infection of the chorioallantoic surface by virus particles diffusing from primary centres of infection. With experience, it is possible to differentiate primary from secondary foci on most membranes when the number of primary lesions does not exceed about 25, but with some there is such a diffuse development of secondary foci that counting is impossible.

Addition of immune serum to egg passage virus reduces the number of foci appearing when mixtures are inoculated. The counts obtained from a series of eggs all inoculated with the same serum virus mixture will, with very rare exceptions, lie within 50 p.c. ± of the mean value, and the degree of reduction from the control count provides a relatively accurate measure of the activity of the serum.

(A) *Time course of the virus neutralization reaction.*

A considerable number of experiments, in which virus-immune serum mixtures were inoculated into eggs at various times after mixing, have been made. The results have varied considerably from experiment to experiment, some showing an approximately constant proportion of survivors, irrespective of the time elapsing between mixing and inoculation, others showing a progressive fall. Our earlier experiments were carried out at room temperature, and have been fully discussed elsewhere (Burnet, 1936b).

When the reagents are maintained throughout at ice-bath temperature, more consistent results are obtained. Fig. 18 shows graphically the results obtained in a recent previously unpublished experiment, using ferret immune serum and egg virus, mixtures being maintained throughout at 0–2° C. and all dilutions made in 10 p.c. normal horse serum saline. This figure, which should be compared with Fig. 14, brings out clearly the influence of serum concentration on the rate at which an equilibrium level is reached. With the strongest serum the proportion of survivors is the same, whether the mixture is tested at once or after an hour's

contact. After two more hours, however, there is a significant fall. Such secondary inactivation is observed in a large proportion of experiments with relatively concentrated serum, and from the results of dilution experiments to be described later must be ascribed to irreversible processes, presumably a firmer union between virus and antibody. More dilute sera require a demonstrable period of time to reach an equilibrium value. Several similar experiments, one of which is illustrated in the paper referred to, have given similar results.

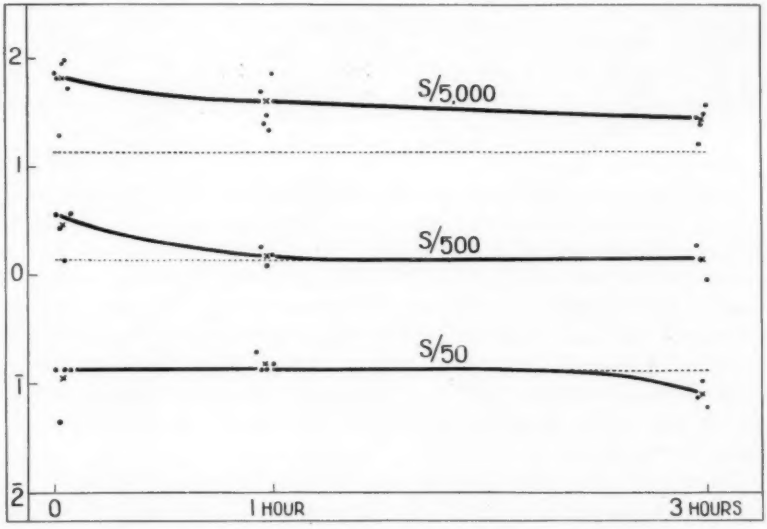

Fig. 18. Time-survivor curves for influenza virus and three dilutions of antiserum. Log. percentage survivors plotted against time. Each dot represents the count of foci from one membrane inoculated at the time shown. x Represents the average value for each group. Dotted lines indicate the theoretical equilibrium values.

In interpreting the time course of the reaction in terms of the results obtained by egg inoculation, it must be remembered that infection of the ectodermal cells does not take place immediately the inoculum is placed on the egg membrane. The number of pocks produced is a measure of the proportion of viable particles at some undetermined time *after* the inoculation has been made. Experiments to determine the average lag before the ectodermal cells are infected have been made by first inoculating virus, then, at varying intervals, adding an excess of antiserum.

In these experiments, the technique of inoculation was slightly modified. After making the triangular window and dropping the chorioallantois, the whole of the exposed shell membrane was clipped away. One drop of virus suitably diluted was dropped vertically on to the membrane. The openings were then shielded with a sterile coverslip, and the eggs replaced in the incubator,

great care being taken to avoid tilting them at all. At the appropriate time, the openings were uncovered and *two* drops of immune serum diluted 1: 10 (i.e. normally sufficient to inactivate undiluted virus completely) dropped vertically, so as to cover the whole area over which the virus could have spread. Incubation was then continued for the usual 40–44 hours after the virus inoculation.

Table 10 shows the result of this experiment.

TABLE 10.

Fixation of Influenza Virus on the Chorioallantois.

Virus dilution.	Immune serum added after: At once.	1 hour.	2 hours.	5 hours.	Control without serum.
1: 10	0, 0, 0	1, 30, 45	++	++	
1: 100			5, 9, 36	24, 30, 40, 55	
1: 1,000				3, 6, 12, 13	14, 16, 16, 16, 18

Figures show the number of specific foci on each membrane.

According to this experiment, fixation of the virus is relatively slow, most of it occurring from 2–5 hours after inoculation. We are doubtful whether this result should be taken entirely at its face value. It may be that virus particles become attached to cells considerably sooner than this, but remain for some time susceptible to inactivation by high concentrations of immune serum. Further, in some instances there may have been complete inactivation of a secondary generation of virus particles, so aborting an incipient focus. Only a few of the membranes, however, showed any indication that the average size of the focus had been diminished by serum, and several showed numerous satellite foci, so that this latter factor is probably unimportant.

The experiment at least shows that there is some lag in initiating the formation of a specific focus, and also that the lag varies with different eggs. It will, therefore, be impossible to evaluate the earliest stages of the reaction between virus and antiserum by experiments of this general type. All we can do is to follow the later stages, realizing that the irregularity of the lag period between inoculation and infection will allow only a rough idea of the course of the reaction to be obtained. A further discussion of the influence of this lag on the results obtained with virus-serum mixtures will be found in Chapter VIII in regard to louping ill virus.

The late secondary fall shown with strong serum in Fig. 18 is of very variable degree. It is, on the whole, much more obvious in experiments with strong serum made at room temperature, but not invariably so, a proportion of such experiments showing a persisting equilibrium level. We have not been able to determine the factors responsible for such differences.

(B) *Relation between serum concentration and proportion of survivors.*

If we take the equilibrium level to which the count of lesions is reduced by immune serum, we find that there is a simple relation between the serum concen-

tration and the percentage reduction. If undiluted serum reduces the count to 0·1 p.c. of the control count, then serum diluted $\frac{1}{n}$ will reduce it to $n \times 0{\cdot}1$ p.c. In other words, if C = concentration of serum and P = proportion of survivors, C.P = constant. When percentage survivors are plotted against the serum concentration, both being expressed logarithmically, the points for each serum lie along a line running approximately at 45°. Fig. 19, taken from Burnet (1936b)

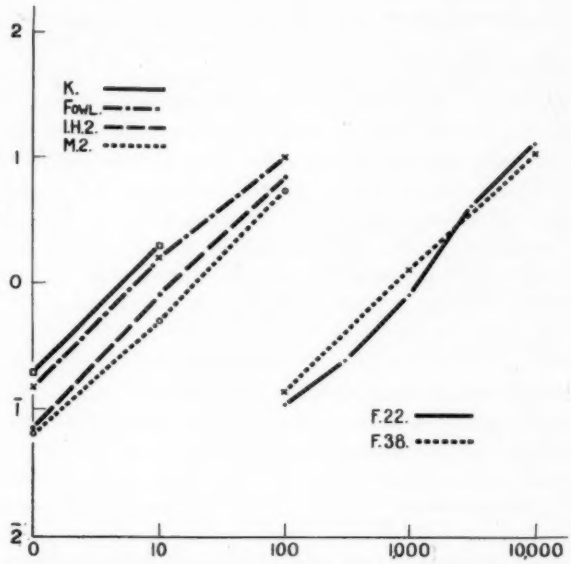

Fig. 19. Serum dilution-survivor curves with influenza virus for several types of sera, to illustrate the approximation to the "C.P. = constant" rule. Two ferret immune sera (F.22, F.38) and one each of human (K), fowl, horse (I.H.2) and mouse (M.2) origin are shown. From Burnet (1936b).

shows the results obtained with a number of different sera. Occasional deviations from the "C.P = constant" relationship have been observed in individual experiments. In one such experiment the time course was also determined, and the deviation was seen to be due to an unusually marked secondary fall with stronger serum, and it is probable that this phenomenon has been responsible for most of the discrepancies.

The relation between original virus concentration and the proportion of survivors with serum concentration constant can be tested only over a narrow range, owing to the difficulty of counting large numbers of foci, and the inadmissibility of testing diluted mixtures (see next section). Within these limitations, the "per-

centage law'' holds, i.e. the proportion of survivors is independent of the original concentration of virus. In the experiments designed to find the proportion of survivors with different serum concentrations, the truth of the percentage law was tacitly assumed.

(C) *Dissociation with dilution of serum virus mixtures.*

If serum virus mixtures are left for a few minutes to reach their primary equilibrium level and then diluted in saline 1 : 10 or 1 : 100, the number of foci obtained from a standard volume of the diluted mixture is the same as that obtained from the original mixture. Since by the ''C.P = constant'' rule, if the mixture V. + S. gives *n* foci, V/10 + S/10 will also give *n* foci, it follows that dissociation is complete under these conditions. If the mixture is kept an hour at room temperature before diluting, the count of lesions obtained from the dilute mixture will be again approximately the same as that of the original tested at the time the dilution was made. If there has been a secondary fall in titre of the original mixture, this will be reflected in the lower count obtained on dilution, indicating that the secondary fall is due to an irreversible reaction. The number of foci obtained from a diluted mixture is the same, irrespective of whether the inoculations are made at once or 30–60 minutes after dilution.

(D) *Interpretation of the influenza virus-antibody reaction on the egg membrane.*

It will be seen that the results with influenza virus approach rather more closely to the ideal case we considered in Chapter V than do the results with vaccinia. In brief, we find that an equilibrium between active virus, antibody and inactivated virus is rapidly reached, the time taken to reach equilibrum being inversely related to the serum concentration, and only experimentally demonstrable when this is low. In the early stages at least the reaction is completely reversible, a new position of equilibrium being established almost immediately when a mixture is diluted. The relationship:

$$\textit{proportion of survivors} \times \textit{serum concentration} = \textit{constant}$$

holds much more generally than was the case with vaccinia experiments.

These results are what would be deduced from theory if a simple dissociable union takes place between antibody and antigenic determinants on the virus particles, either by using Langmuir's relationship, or from mass action considerations. For discussion of this, Burnet's (1936b) paper may be consulted.

The secondary irreversible reaction postulated for vaccinia is here more directly demonstrable, since reactions in which it occurs can be compared with those in which the reversible equilibrium is maintained. The conditions responsible for these two different types of time course, however, have not been determined.

Compared with vaccinia virus, the immunological reactions of influenza virus are more clear-cut. It is a better antigen, in the sense that the average convalescent

serum is active to a much higher dilution, and if we can judge from the regularity of the results, the population of virus particles is more uniform in susceptibility to antibody.

2. Antibody Absorption Experiments with Influenza Virus.

In contrast to most other viruses which have been studies, it is not difficult to remove a considerable proportion of antibody from an anti-influenza serum by absorption with virus-containing tissue followed by heat inactivation of residual virus. A series of experiments has been carried out, in which immune sera of ferret, horse and human origin have been absorbed with infected mouse lung or egg membrane. The absorbed serum has been compared with serum similarly treated with uninfected tissues by titration of antibody content, both on the egg membrane against egg passage virus and in the mouse lung against mouse passage virus. The results have shown (1) that 90 p.c. or more of the antibody demonstrable by the egg membrane technique can be easily removed with either egg or mouse tissue virus; (2) that in all sera absorbed with egg virus, and in most of those absorbed with mouse virus, there is no significant reduction in the antibody content as estimated by mouse titration; and (3) that with some sera, absorption with large amounts of infected mouse lung does reduce the antibody content as estimated both by mouse titration and by titration on the egg membrane.

In addition to our standard egg and mouse passage strains, we have available a strain pathogenic for both species, but in each respect slightly less virulent than the "monovalent" strains. The strain was developed from a well adapted egg passage strain by a short series of mouse passages followed by irregularly alternating mouse and egg passages. It is now fairly stable in virulence and appears to be composed of uniformly active particles rather than a mixture of egg virulent and mouse virulent particles. Subinoculation of the strain to mice after several egg passages, including some in which limiting dilutions were used, gave the same typical lesions as when mouse lung virus of this strain was used.

In connection with the present problem of why egg and mouse titrations show such gross differences in the degree to which a serum is apparently absorbed, such a strain has obvious uses. Since it can be titrated in both indicator organisms, any differences observed must be related to processes occurring *in vivo*, not to any physicochemical difference between egg and mouse viruses which can manifest its effect *in vitro*.

Table 11 shows that when the *same mixtures* of absorbed serum with E.M. virus are inoculated into eggs and mice, quite different degrees of absorption of antibody are indicated. In the mice, approximately one-third of the antibody appears to be still active, i.e. there is a distinct reduction as compared with the completely negative result obtained with mouse virus titration. But on the egg membrane only one per cent. of the original antibody can manifest activity. This is even more marked a reduction than is shown with egg virus.

TABLE 11.

Comparison by Various Titration Methods of Serum F.50 Unabsorbed and Absorbed with Egg Virus.

1. *Egg membrane titrations.*

Strain.	Unabsorbed serum 1: 100.	Absorbed serum 1: 100.	Reduction in antibody activity.
Egg virus	0·082% foci	2·65% foci	96·9% reduction
E.M. virus	0·4% "	43% "	99·1% "

2. *Mouse titrations.*

Strain	Unabsorbed serum.			Absorbed serum.		Reduction in antibody activity.
	1: 100	1: 300	1: 1,000	1: 100	1: 300	
E.M. virus	0, 0, 0	1, 1, 2	3, 4, 4	1, 1, 2	2, 4, 4	67% reduction
	1: 100	1: 400				
Mouse virus	0, 1, 1	4, 4, 4		0, 0, 1		nil

Mouse lung lesions indicated: 0 No consolidation.
1, 2, 3 Increasing proportions of consolidation.
4 Complete consolidation and death.

This experiment shows clearly that the factor responsible for the differences between egg titrations and mouse titrations is the nature of the susceptible tissues, and not any difference in the reactions *in vitro*. E.M. virus particles partially coated with antibody of absorbed serum can enter embryonic chick cells and provoke typical foci, but are unable to initiate lesions in the mouse lung. In many respects we can compare the activity of absorbed influenza serum in egg and mouse with that of a normal antivaccinia serum in egg and rabbit skin. In each case the serum is much more effective in the grown mammal than in the embryo chick. We have already discussed the possible significance of locally retained antibody and early actively produced antibody in influencing the rabbit skin reactions to vaccinia virus-serum mixtures. In the present instance, neither of these factors seems to be of much importance. Antibody intratracheally inoculated is not likely to persist long in the mouse lung, and direct experiment showed no appearance of active immunity until the seventh day after intranasal inoculation. The essential difference may well be a more subtle one.

Under normal circumstances, the chick embryo lives in a sterile, completely sheltered environment, into which micro-organisms cannot penetrate. Until the time of hatching approaches, there is no biological necessity for any mechanism to deal with infection immunologically or in any other way. The work of Murphy (1913, 1914) on the growth of the Jensen rat sarcoma on the chorioallantois is very illuminating in this regard. He found that the foreign sarcomatous tissue proliferated readily up to the 19th day of incubation without provoking any inflammatory response from the adjacent chick tissues. But on the last two days of incubation a marked cellular reaction appeared, and the foreign cells underwent degeneration and necrosis. It was as if, when the organism appreciated the

imminence of an unsheltered existence, a mechanism came into existence to recognize and react against foreign material in its tissues. Now the only foreign protein that "normally" reaches an animal cell is of micro-organismal origin, and this reaction against rat sarcoma cells probably signifies essentially that a mechanism developed to deal with micro-organisms has come into action on the 20th day of incubation. Our 12–14 day embryos then have no adaptations to deal with microbial invasion, but mice, like other free living animals, have adequate mechanisms for dealing with such a "normal" emergency. Even if, as seems likely, a single influenza virus particle of a properly adapted strain is sufficient to initiate a lesion either in mouse or embryo, it is unlikely that the same degree of antibody coating will diminish infectivity equally for both. It is only necessary to give the mouse lung cell a slight advantage over the virus to prevent infection, but for the embryo cell it may be necessary that the virus particle should be almost completely coated by antibody if infection is to be averted.

The majority of the experiments show absorption of "egg" antibody and no absorption of mouse antibody, irrespective of whether egg or mouse virus is used for absorption. With some sera, however, absorption with mouse virus removed "mouse" antibody as well as "egg" antibody, although absorption with egg virus has never removed "mouse" antibody. Table 12 shows the results obtained with serum F.50 from a ferret inoculated intranasally with mouse virus.

TABLE 12.

Antibody Absorptions with Serum F.50.

Absorbed with:		Reduction in antibody as shown on: Egg membrane.	Mouse lung.
Egg virus	1	93%	Nil
	2	85%	"
	3	92%	"
	4	95·6%	"
	5	96·9%	"
Mouse virus	1	82%	90%
	2	92·8%	90–95%
	3	89·6%	66%
	4	80·3%	Nil

Note. Mouse virus absorptions 2, 3, and 4 were made with successively smaller doses, 3·5, 1·5 and 0·5 mouse lungs respectively.

This was the only one of four ferret immune sera to show absorption of mouse antibody. One of three human immune sera and the immune horse serum I.H.2. of Laidlaw, Smith, Andrewes and Dunkin (1935) also showed definite reduction of mouse antibody on absorption with infected mouse lung. In a few experiments, no absorption of either type of antibody was obtained, notably with a pooled immune mouse serum.

The results with serum F.50 are of particular interest, since they indicate that with the changes in virulence associated with passage on the different susceptible

species, certain changes in the antigenic determinants on the virus surface have occurred. Another indication of the interrelation between virulence and immunological behaviour is provided by the fact that the strain E.M., which is much less pathogenic for the chick embryo, is also less readily inactivated by immune serum (see Table 11). In view of these fine differences in antigenic behaviour amongst the derivatives of a single strain of virus, a study of strains of different origin has been begun, but the work is too incomplete to justify including the results here.

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Chapter VIII.

LOUPING ILL VIRUS.

Animals recovering from louping ill are solidly immune to re-infection, and their blood can be shown to contain antibody by suitable tests. Schwentker, Rivers and Finkelstein (1933) immunized monkeys by intraperitoneal inoculation and tested their sera by a method using intra-cerebral inoculation in mice. Serial dilutions of virus were mixed with equal volumes of undiluted serum and inoculated after three hours' contact. The method was used by Rivers and Schwentker (1934) to demonstrate that certain laboratory infections in man were due to this virus. Little theoretical work on the nature of the reaction has been done. Goyal (1935) reported experiments indicating that a certain amount of reactivation of neutral mixtures could be obtained by methods similar to those used with vaccinia by Andrewes.

1. Neutralization on the Chorioallantoic Membrane.

Only a brief mention was made of the possibility of using the egg membrane for neutralization tests with louping ill virus in a paper dealing with the general nature of the virus lesions in this situation (Burnet, 1936b). At that time we were doubtful of the practicability of titrating the virus on the egg, owing to the difficulty of distinguishing between primary and secondary foci. On returning to the problem we found that, possibly on account of certain changes in the method of preliminary incubation, and with increased experience in the counting of "difficult" membranes, much more satisfactory results were obtained. A large series of detailed experiments was made, and a clear picture of the kinetics of the reaction has emerged. Louping ill virus is much the smallest (17 mμ in diameter) of those with which we have worked, and in many respects the results approach more closely to our "ideal case" than those with any other virus. Since no account of this work has been published, we are presenting it with rather more experimental detail than has been given in other sections.

2. Susceptibility of the Chorioallantois to Infection.

It was previously noted that the egg membrane was a more sensitive indicator of virus than the mouse brain. A detailed comparison of the two methods of titration, using egg passage virus, was made to confirm this point, and incidentally to establish whether one focus could be taken to represent the minimal infecting dose for the embryo. With influenza virus (Burnet, 1936a) it had been found that the number of foci was about half the number of M.I.D.'s With this object, in addition to the normal titration series, ten eggs were inoculated with 0·05 c.c. each of the $1:10^6$ dilution and allowed to incubate for five days. The liver of each embryo was removed, emulsified in broth, and inoculated intracerebrally in two mice. Seven

embryos gave liver emulsions killing both mice with typical symptoms in five to six days: the other three were negative. Table 13 shows the combined result of the experiment. Since the dose inoculated intracerebrally, 0·025 c.c., was half that used for eggs, it will be seen that, assuming one focus corresponds to one virus particle, 30 virus particles inoculated intracerebrally killed two out of three mice, while three particles were innocuous. This result is of the same order as was found in previous experiments, in which virus in the form of mouse brain was titrated by both methods. The proportion of infected embryos in the limiting dilution corresponds closely with the pock count in the next dilution. This result suggests strongly that the egg membrane responds by the formation of a focus to almost every virus particle inoculated, and that the pock count can be taken as an approximate measure of the absolute amount of virus inoculated.

TABLE 13.

Comparative Titration of Louping Ill Virus (Egg Membrane Strain) on the Chorioallantois and by Intracerebral Inoculation in Mice.

Test Animal.	Dilution of stock virus			
	1: 10^3	1: 10^4	1: 10^5	1: 10^6
Eggs		64, 40, 20+, 53	8, 10, 4, +	7/10 infective for mice
Mice	†6, †8	†8, †12, S	S, S, *3	S, S, S

Notes. Results of egg membrane titrations are given as numbers of foci on membrane removed two days after inoculation, except for the dilution 1: 10^6. The ten eggs inoculated with this dilution were incubated for five days, and the embryo liver tested for the presence or absence of virus by intracerebral inoculation in mice with the result shown.

Results of mice inoculations: † death with typical symptoms on day shown.
* death from nonspecific cause on day shown.

3. NEUTRALIZING POWER OF ANTISERA.

In these experiments, immune monkey sera only were used. Two of these had been stored in the cold for about three years, and were obtained through the kindness of Dr. T. M. Rivers and Mr. I. A. Galloway respectively. Most of the experiments were done with these two sera, which were of almost exactly similar neutralizing power. In order to determine whether freshly prepared antiserum had the same properties, two monkeys were immunized according to the method of Rivers and Schwentker (1934) with three intraperitoneal injections of living virus, followed by a test intracerebral inoculation. Neither showed any symptoms, and they were bled ten days later.

Tested immediately, the pooled and filtered serum from these monkeys was rather more active than the older sera, undiluted serum reducing the pock count of an equal volume of virus to 0·0016 p.c. of the original, compared with about 0·003 p.c. for the other sera. After a month in the refrigerator, its value fell to 0·06 p.c., i.e. it had less than 1/30 of its original activity.

4. The Time Course of Louping Ill Neutralizations.

A large number of experiments were done on the time course of the reaction between louping ill virus and the two older sera R. and G., and two experiments were made with the recent serum M. The procedure used was to prepare a suitable mixture, usually egg virus, diluted 1 : 3, with serum, also diluted 1 : 3, using ice cold reagents. As soon as the mixture had been thoroughly shaken, previously opened eggs were inoculated with 0·05 c.c. volumes, and the inoculations repeated

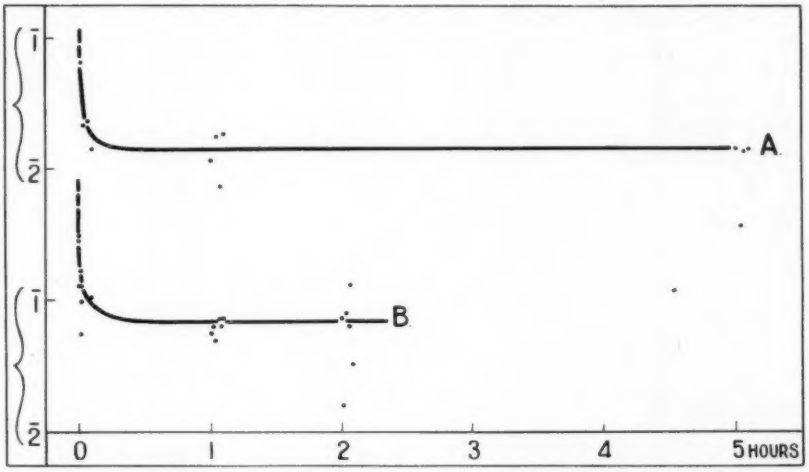

Fig. 20. Time-survivor curves for louping ill virus neutralization at ice-bath temperature. A: with serum R. diluted 1: 6. B.: with serum M. diluted 1: 2. Each dot represents one membrane count.

at the required intervals. Eggs were incubated at 38·5° C. and opened after 40–48 hours' incubation. The results of representative experiments are shown graphically in Figs. 20 and 21. Each membrane count is represented by a dot showing the logarithm of the percentage of survivors against the time after mixing at which the egg inoculation was made. A few experiments made at room temperature gave results of the same general character, but the reduction was greater at the higher temperature. In one direct comparison, at room temperature 34° C. (Australian summer!), early counts at 1–5 minutes averaged 0·026 p.c. survivors, falling to 0·0045 p.c. at one hour, while the corresponding figures at 0–3° C. were 0·056 p.c. to 0·014 p.c.

Curves A. and B., Fig. 20, show the results obtained with sera R. at a final dilution 1 : 6; and M, at 1 : 2, the mixtures being kept in an ice bath throughout.

All completed experiments, using relatively concentrated serum, showed essentially similar findings, i.e. a relatively high initial count with a fall to an equilibrium level at one hour, this level being maintained thereafter. In an experiment

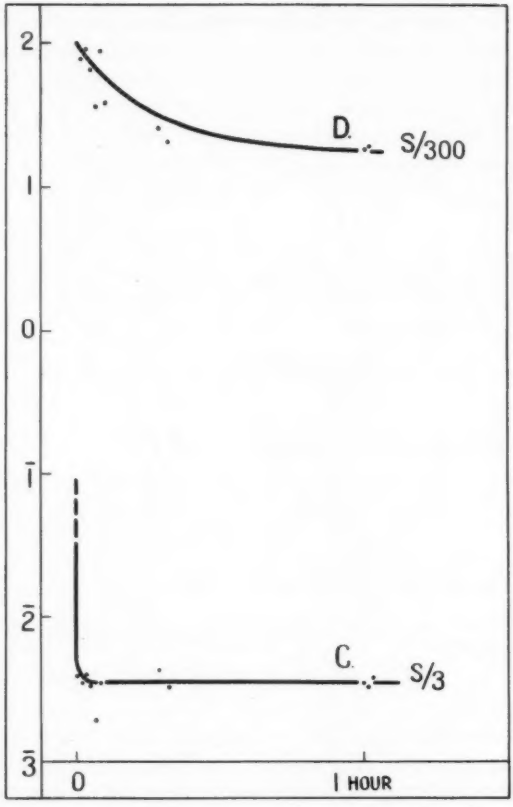

Fig. 21. Time-survivor curves for louping ill virus neutralization with serum R. 1: 3 and 1: 300 at room temperature.

which was prolonged to 24 hours, the following pock counts were obtained: 1–5 minutes, 62, 70, 84, 50; 1 hour, 18, 28, 11, 27; 5 hours, 26, 24, 7, 14; 24 hours, 4, 30, 8, 17. The very slight additional fall at 24 hours was also present in the controls with normal serum, the average count falling from 41 to 29.

Curves C. and D., Fig. 21, show the results of two experiments carried out at room temperature (about 20° C.). Curve C. with strong serum shows no time

effect at all, the counts being remarkably consistent throughout. Curve D. shows the action of serum diluted 1 : 300 in an experiment made under the same conditions. Here the fall continues to one hour, and would probably have gone further had the time of the experiment been extended.

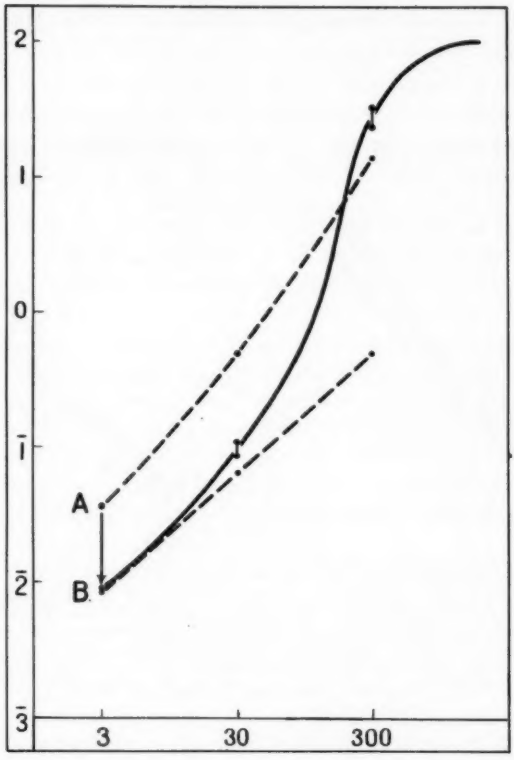

Fig. 22. Serum concentration-survivor curve for louping ill virus neutralization. Two concordant experiments combined to give the heavy line. Dotted lines from A. and B. show the results of early and late dilution experiments carried out along with one of these determinations.

5. Serum Concentration-survivor Curves.

The serum concentration-survivor curves of the three sera are very similar. All show an approximate 45° slope from undiluted serum to 1 : 30. Higher dilutions of serum have proportionately much less action (in the standard time of one hour), and the curve rises steeply (Fig. 22). There has been some variation in the absolute percentages of survivors from experiment to experiment, but the shape of all curves obtained has been almost identical.

6. DISSOCIATION BY DILUTION.

The louping ill virus-antibody reaction is freely, and probably completely, reversible. Most experiments on the subject have been made with our standard mixture serum 1 : 3 + stock egg virus 1 : 3. Immediately after a preliminary test of the undiluted mixture on eggs has been made, 1 : 10 and 1 : 100 dilutions of the mixture are made in normal serum saline, shaken, left for 10–15 minutes, and then inoculated in eggs. The same procedure is also carried out after the mixture has

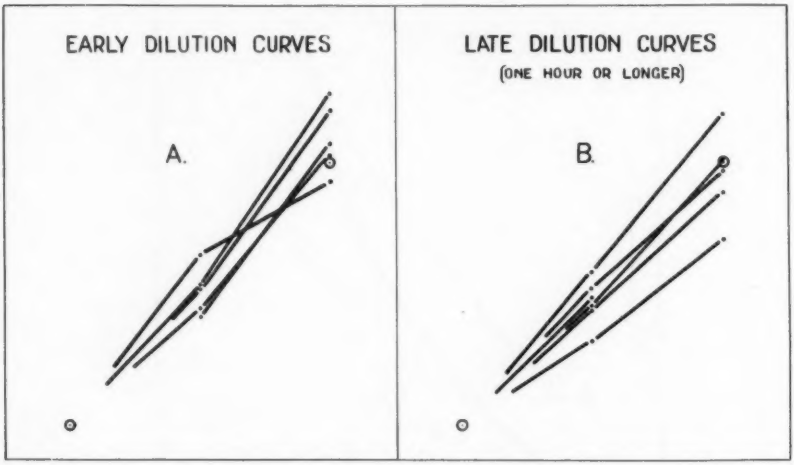

Fig. 23. Results of all completed dilution experiments in which strong serum virus mixtures were diluted 10 and 100 fold (A) within a minute or two after mixing, (B) after contact for one hour or longer. A straight line joining the two circles would represent the result if the serial dilutions of the mixture gave exactly the same number of foci as the original. Late dilutions show a general approximation to this. Early dilutions give on the whole significantly greater numbers of foci than the original mixture.

been held for 1, 2, or 5 hours. In Fig. 23 we have combined the "early" (2–10 minutes) and "late" dissociation experiment results, five complete experiments of each type being shown. The percentage of survivors, when the undiluted mixture is tested, is taken as the unit in all curves and the results obtained on dilution plotted logarithmically in terms of this value. To take an example, if undiluted mixture gives an average count of ten foci, corresponding to 0·01 p.c. of survivors, a typical early dilution result would be to obtain from the 1 : 10 dilution eleven foci (= 0·11 p.c.) and from 1 : 100 dilution twenty foci (2·0 p.c.). These points are plotted logarithmically as: 0, 1·04 and 2·30, and joined by straight lines.

It will be seen that, with some variability, early dilution experiments give a curve significantly steeper than 45°, especially for the hundredfold dilution,

while "late" dilution experiments give results close to, and falling on either side of, the 45° axis. Typical dilution curves made in the same experiment are also superimposed on the serum concentration-survivor curve (Fig. 22) to help make the relations clearer.

7. Interpretation of Time Relationships in Neutralization with the Egg Membrane Technique.

The experimental data in regard to time relationships are fuller for louping ill virus than for the other viruses with which we have worked, and it has seemed better to introduce a discussion of the subject here rather than in Chapter V. As far as the limitations of titration accuracy allow us to determine, louping ill neutralizations correspond to the "ideal case" of our discussion there. Fig. 24 has been constructed according to the hypotheses involved in the "ideal case" for a serum which, undiluted, reduces the survivor count to 0·01 p.c. of the original, diluted 1 : 10 to 0·1 p.c., and so on. The solid line represents the "real" time course of the reaction (S/1 + V).

When we place an "immediate" sample of the mixture on the chorioallantois, there will be a certain lag before any effective contacts with cells take place. Some particles will take longer than others, but we may assume that 90 p.c. of contacts take place in the period T1 — T2. The reaction is meantime proceeding, and the range of counts to be expected from "immediate" inoculations will be that corresponding to the range R1, through which the curve of neutralization passes during the time T1 — T2. Inoculations made once the equilibrium level has been reached will naturally give constant results.

The time taken to reach the equilibrium level will be inversely related to the serum concentration. The standard one hour (+ T1) allows equilibrium to be reached by serum dilutions up to 1 : 30 with the sera R. and G., but beyond that the time is insufficient, and we obtain the typical steep upper portion of the serum concentration-survivor curve (Fig. 22, cf. Curve B. in Fig 15).

In dilution experiments we can intercept the early stages of the reaction without having to allow for a lag period. If dilutions are made before the reaction has reached the level characteristic of S/100 serum mixtures, ten and one hundred-fold dilutions will follow the courses shown. The number of "free" virus particles will diminish toward the new equilibrium levels at proportionately slower rates, and inoculations made soon after dilution will give the steeper curves such as are shown in Fig. 23A. Incidentally, we may note that this ability to intercept the reaction effectively by dilution at earlier stages than are possible by any direct inoculation method (all involving some lag before cells can be infected) allows "time effects" to be readily demonstrated in all but one of the virus-antiserum reactions which have been studied. With vaccinia, for example, it was pointed out that, while direct titrations of mixtures with strong serum showed very little indication

of change in average count with time, if one diluted a mixture immediately after it was made and again an hour later, tests at the two diluted mixtures showed the former to be much more active. Similar findings have been recorded for a wide range of viruses.

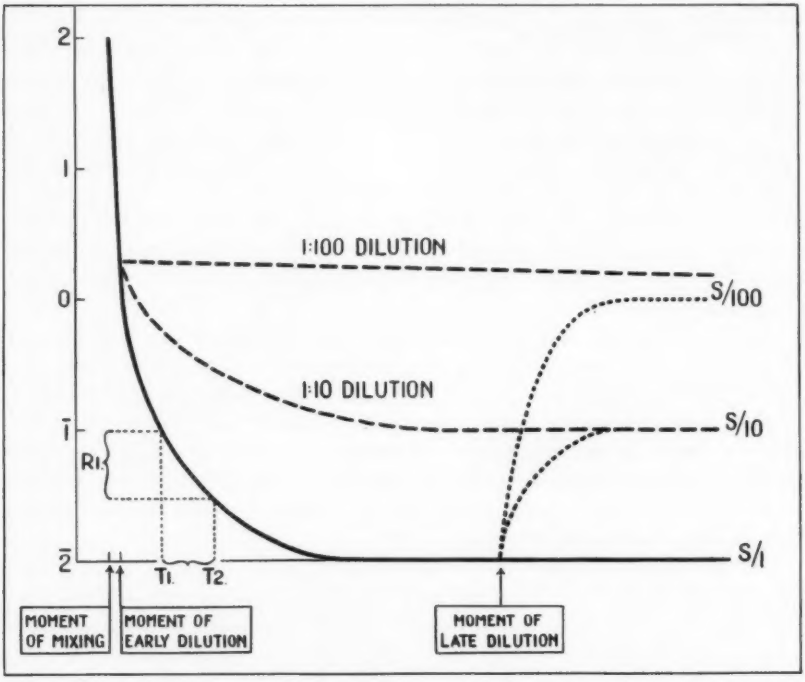

Fig. 24. A diagram in the form of a time-survivor curve to illustrate the theoretical basis of dilution experiments with louping ill virus and antiserum. (See text.)

Dilutions made when the mixture has reached equilibrium have merely to reach a new equilibrium level by a dissociation process which will allow equilibrium to be approached much more rapidly than if it had to be reached from the opposite side. Theoretically, therefore, late dilution experiments should provide a 45° curve.

8. Summary.

This detailed correspondence between the experimental results with louping ill virus and the implications of our *a priori* discussion would seem to indicate that our simplifying assumptions are more nearly valid for this virus than for any

of the others we have studied. We have considered the possibility that our results have been unconsciously biased to conform to a preconceived theory, since with many membranes there is room for considerable difference of opinion in assessing the number of primary foci. This possibility can, we think, be eliminated with certainty, since the theory in question was in a very nebulous form at the time the experiments were done, and we were at a loss to account for features such as the difference between early and late dilution curves and the complete absence of a time effect in some experiments, the significance of which became apparent later.

Accepting the results as genuine, the concordance with theory would indicate (a) that louping ill virus particles are particularly uniform, (b) that a very small number of antibody molecules (perhaps only one) united to the virus particle suffice to inactivate it, (c) that the reaction with antibody is wholly reversible, and (d) that chorioallantoic ectoderm cells are uniformly susceptible to the virus.

The uniformity of character and the small number of antibody molecules required to inactivate would be expected from the small size of the virus particle (15–20 mμ), and the high susceptibility of the chorioallantois has been directly demonstrated above.

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Chapter IX.

RIFT VALLEY FEVER.

Findlay (1936) studied the inactivation of Rift Valley fever virus by human immune serum. He was concerned chiefly with the phenomenon of reactivation of "neutral" mixtures by dilution. Table 14, modified from his paper, shows a typical result when mixtures of undiluted immune serum and serial dilutions of virus were allowed to stand four hours at 37° C. and then diluted serially in saline before inoculation in 0·4 c.c. quantities by the intraperitoneal route.

Table 14.

Reactivation by Dilution with Rift Valley Fever Virus (Findlay).

Original mixtures. Undiluted serum + virus diluted	Subsequent dilution after 4 hours at 37°C.				
	Undil.	10	10^2	10^3	10^4
1: 10	2/4	4/4	4/4	4/4	4/4
10^2	0/4	3/4	4/4	4/4	4/4
10^3	0/4	2/4	4/4	3/4	2/4
10^4	0/4	1/4	4/4	1/4	0/4
10^5	0/4	0/4	0/4	0/4	0/4

Virus diluted with normal serum active to 1: 10^9.

Figures show number of mice dying/number inoculated.

It will be seen that in mixtures containing serum 1 : 2, and sometimes 1 : 20, there is complete or partial protection which is not evidenced in the immediately higher dilutions.

In order to apply the ideas developed in previous sections to the very complete data supplied by Findlay, it is advisable to transfer his results, e.g. those in his Table 4, p. 95, to the standard serum concentration-survivor curve form. This has been done in Fig. 25 on the basis that if the highest dilution of a mixture causing any deaths shows 4/4 deaths, each dose is taken to contain one surviving particle, 2/4 deaths 0·5 particles, and so on. Curve A. (heavy line) shows the results when, as in our experiments, initially diluted serum and virus are mixed, and, after a standard time of contact, inoculated intraperitoneally; curve B. shows the results when mixtures containing initially undiluted serum (i.e. final dilution 1 : 2) are diluted after the same time of contact to give the serum concentration shown.

Put in this form, the resemblance to our results with louping ill is close. We should regard the true equilibria corresponding to the different serum concentrations to be represented by the curve B., which runs almost precisely at an angle of 45° (i.e. C.P = constant), just as do "late dilution" curves from louping ill ex-

periments. When we are dealing with equilibria of the present type with very dilute reagents, a new equilibrium level is much more rapidly reached when only dissociation processes are necessary (i.e. dilution from an equilibrium with strong serum) than when union between sparsely distributed entities has to take place

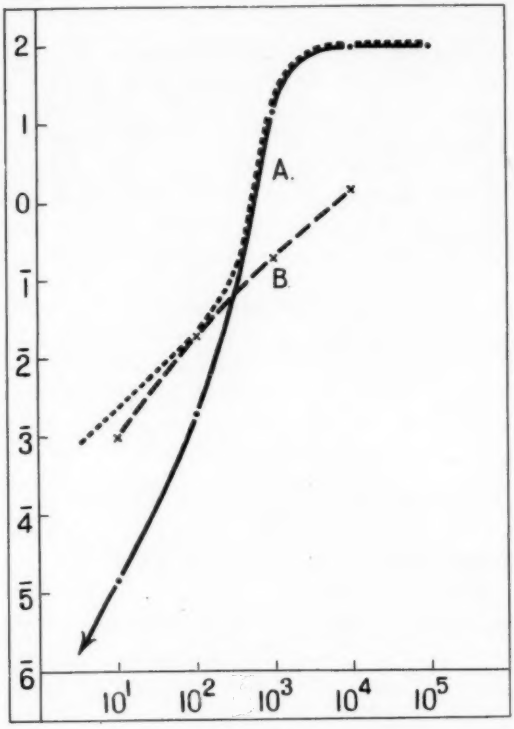

Fig. 25. An analysis of Findlay's results with Rift Valley fever virus in the form of a serum dilution-survivor curve. A. (heavy line): results with mixtures of previously diluted reagents. B. (broken line): result of inoculation of dilutions made from initial mixtures of virus and undiluted serum. C. (dotted line): the "real" serum concentration-survivor curve (see text).

(attainment of equilibrium by initially diluted reagents). The shape of curve A., then, is, on this hypothesis, due in part (i.e. in regard to the portion above curve B.) to insufficient time being allowed for equilibrium to be attained.

The lower portion of curve A., which corresponds to the region in which reactivation by dilution is possible, is depressed below what we would regard as the equilibrium level, owing to the continuing action of relatively large amounts of

serum inoculated intraperitoneally. The conditions are quite comparable to those we discussed in regard to vaccinia in intradermal tests. When a much smaller dose, 0·03 c.c., of the mixtures is given, the serum is diluted by body fluids beyond the point at which the postulated inactivating effect on secondary generations of virus is effective. It is possible that early active production of antibody in mice receiving mixtures containing large amounts of virus may also play a part, as Findlay suggests.

On the figure we have indicated what we regard as the "real" serum concentration-survivor curve, i.e. curve B. up to the point at which it cuts A., and then curve A. Comparison with the corresponding curve for louping ill virus will show that they are practically identical. This is in conformity with the general resemblance between the two viruses—both are small, both capable of infecting the central nervous system, but present in the blood and being spread naturally by blood-sucking arthropods. The range of susceptible species—sheep, man, monkey, mouse—is the same, and both viruses provoke a solid immunity after infection.

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Chapter X.

EQUINE ENCEPHALOMYELITIS VIRUS.

The *in vitro* reactions of this virus with homologous antiserum have recently been studied in detail by Merrill (1936a and b) with results which, having regard to the different method of titration used, correspond closely to those obtained with the viruses we have studied on the chorioallantois. He used as a standard method mixture of serum and virus dilutions in the ice bath with inoculation of 0·03 c.c. amounts intracerebrally in mice as soon as possible after mixing. Under these conditions there will be the least likelihood of secondary reactions playing a significant part.

1. Percentage Law.

Within the limitations of the tenfold titration method used, and the relatively weak antiserum, the proportionate reduction effected by serum was independent of the concentration of virus. Undiluted serum mixed with an equal volume of diluted virus reduced the titre by 90–99 p.c.

Table 15.

Serum-Virus Dilution Experiments with Equine Encephalomyelitis Virus (Merrill).

	Intracerebral Tests.			Intraperitoneal Tests.		
	Virus and immune serum		Virus and normal serum	Virus and immune serum		Virus and normal serum
Dilution of serum-virus mixture.	Mixed and diluted.	Diluted and mixed.	Mixed and diluted.	Mixed and diluted.	Diluted and mixed.	Mixed and diluted.
Undiluted	—	—	—	0/5	0/5	—
10^{-1}	—	—	—	0/5	0/5	—
10^{-2}	—	—	—	0/5	0/5	5/5
10^{-3}	3/3	—	—	0/5	3/5	4/5
10^{-4}	4/5	5/5	5/5	0/5	4/5	4/5
10^{-5}	1/5	5/5	5/5	0/5	1/5	2/5
10^{-6}	0/5	3/5	2/5	—	—	—
10^{-7}	—	0/5	1/5	—	—	—

Results are shown as number of mice dying/number inoculated.

2. Dissociation on Dilution.

When undiluted virus and serum were mixed and the mixture subsequently diluted serially, more protection was evident with the higher dilutions than was shown if mixtures containing the same final dilutions of serum and virus were

prepared from initially diluted components. Table 15 contains the result of one such experiment, the form of the table being somewhat modified from Merrill's. It is not possible to judge from the data whether this difference is due to (1) irreversible inactivation of a proportion of the virus particles by strong serum, or (2) an insufficient time being allowed to elapse for the very weak serum concentrations to reach an equilibrium condition. Although the experiment undoubtedly proves Merrill's contention that *in vitro* reaction between antibody and virus occurs, we should favour the second explanation rather than the first, towards which he leans.

3. THE "C.P = CONSTANT" RELATIONSHIP.

Experiments on the inactivating power of different concentrations of serum summarized in Merrill's Table 4 (p. 198 of his paper) showed a very close approach to the "C.P = constant" relationship. Undiluted serum gave almost exactly a thousandfold reduction in infectivity, 1 : 10 serum a hundredfold, and 1 : 100 serum slightly more than a tenfold reduction.

4. INFLUENCE OF ROUTE OF INOCULATION ON APPARENT ACTIVITY OF SERUM.

Immune serum-virus mixtures were much less infective when given intraperitoneally than when inoculated intracerebrally. Serum diluted 1 : 10 was capable of neutralizing undiluted virus when mixtures were given by this route, but could neutralize only a 10^{-6} concentration as judged by intracerebral tests. The resemblance to the conditions discussed in regard to the comparative activity of vaccinia immune serum in the rabbit skin and on the chorioallantois is obvious.

5. BLOCKING OF ANTIGENIC POWER BY IMMUNE SERUM.

Cox and Olitsky (1936), in a series of papers on the active immunization of guinea-pigs against this virus, made some interesting observations on the influence of immune serum in preventing the antigenic effect of formolized virus which bear on the nature of virus-antibody interaction. They had found that subcutaneous injection of formolized virus, equivalent to 3×10^7 infecting units of active virus, on two occasions at a week's interval induced solid immunity in guinea-pigs. In these experiments 1 c.c. of hyperimmune guinea-pig serum at the required dilution was injected one hour before each immunizing injection of formolized virus. The immunity test given 20 days later consisted of an intracerebral inoculation of about 1,000 M.I.D. It was found that serum undiluted, or diluted 1 : 4, prevented any antigenicity being manifested. With 1 : 8 and 1 : 16 serum, a proportion of immune animals resulted; with higher dilutions the antigenic action of the formolized virus was not detectably altered. Very similar results were obtained with active virus in similar experiments.

This provides a clear demonstration of the action of immune serum on virus *in vivo*. Two possible explanations of the phenomenon present themselves: (1)

Virus particles coated with antibody *in vivo* are taken up by cells incapable of producing antibody in response to ingested antigen material. It is possible that while uncoated dead virus particles are predominantly taken up by reticulo-endothelial cells capable of antigenic response, antibody coated particles are predominantly taken by polymorphonuclear leucocytes which are generally presumed not to be concerned in antibody production. (2) Virus particles whose antigenic determinant groups are blanketed with antibody cannot function as antigens, even when they are ingested by a cell normally capable of antibody production. There is practically no evidence available as to the fate of virus particles which enter a cell, but, either because of intrinsic lack of virulence or because they are coated with antibody, do not succeed in multiplying therein. Presumably they are eventually broken down by intracellular enzymes.

In either case it appears that the fragments resulting from intracellular digestion are incapable of acting as antigens producing active immunity to infection, and so far as it goes this evidence of Olitsky and Cox points to the necessity for the virus particle to be intact (even though dead) if it is to function as an antigen. However, we know so little about the processes between the introduction of antigen and the production of antibody that no great weight can be placed on the evidence.

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Chapter XI.

NEWCASTLE DISEASE VIRUS.

It has not been found practicable to develop a pock-counting method for titration of this virus on the chorioallantoic membrane. This is probably due solely to the rapidly lethal nature of the infection induced—inocula which are infective at all produce death almost invariably within 48 hours. The developing embryo can, therefore, only serve, like any other experimental animal, as an indicator of the presence or absence of a lethal dose of virus (Burnet and Ferry, 1934).

It is not difficult to immunize fowls with the Australian strain of virus, and such fowls provide relatively active immune sera. Undiluted, they protect against 1,000 minimal infective doses for the developing embryo, and show some protective power to 1 : 1,000 dilution.

In general, the results obtained by Keogh (unpublished) ran parallel to those obtained with viruses like influenza and louping ill, but the less accurate titration method rendered it impossible to recognize any small deviations from the ideal case. Table 16 shows the result of a serum titration which indicates a fair agreement with the "C.P = constant" rule.

Table 16.

Neutralization of Newcastle Disease Virus by Serum.

Serum dilutions.	Virus dilutions						
	Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Undiluted	†S	SS	SS	SS	SS		
10^{-1}	††	††	†S	SS	SS		
10^{-2}	††	††	†S	†S	SS		
10^{-3}		††	††	†S	SS		
10^{-4}				††	†S		
Broth					††	SS	†S

† Death of embryo.
S Survival of embryo for 3 days.

No time effect could be demonstrated, and dilution experiments gave results indicating that the reaction was completely reversible. A comparison was made between mixtures prepared (a) by mixing undiluted serum with virus in serial dilutions and diluting 1 : 10 before inoculation, and (b) diluted to the corresponding degree before mixing. No significant difference was detected either with egg inoculations or by intramuscular inoculations in three to four-day chicks.

A point of interest which arose in these experiments was the increased survival time of embryos in eggs which were inoculated with virus and some time later received a second inoculation with immune serum. Table 17 shows that under these

conditions the survival time was increased from less than two days to three or four days. If the virus inoculum is small, countable discrete lesions, very similar to those produced by influenza virus, appear on the membrane. Their numbers correspond roughly to what would be expected from the titre of suspensions estimated by the minimal lethal dose. Unfortunately the elaboration of the technique with repeated handling of the eggs is reflected in marked variation in the counts on different membranes, so that the method presents no advantages for titration. It is to be noted, however, that eggs inoculated under these conditions, i.e. where the virus has gained access to the cells prior to the serum, invariably die, though they survive much longer than controls with minimal doses without serum. It will be noticed that the amount of undiluted serum added was much more than sufficient to protect completely against the 10^{-4} dilution of virus when mixed before inoculation. Thirty minutes' delay was, therefore, sufficient to allow at least a proportion of virus particles to infect cells before they were exposed to the immune serum.

TABLE 17.

Influence of Time of Administration of Serum on Period of Survival.

Inoculum.	Interval between virus and serum.	Result.				
Virus 10^{-2} + S	Together	S	S	S	S	†3
„ 10^{-3} + S	„	S	S	S	S	S
„ 10^{-4} + S	„	S	S	S	S	S
„ 10^{-4} + S	30 minutes	†3	†3	†3	†4	†4
„ 10^{-4} + S	1 hour	†3	†3	†4	†4	†4
„ 10^{-4} + S	2 hours	†3	†3	†4	†4	†4
„ 10^{-4}	No serum	†2				
„ 10^{-5}	„	†2				
„ 10^{-6}	„	S				

Attempts to analyse the cause of delayed death and the associated development of well-marked focal lesions have not led to definite conclusions. *A priori* considerations would suggest that the presence of passively supplied antibody in the embryo fluids would tend to prevent generalization and confine the spread of infection to cells immediately contiguous to the initial lesion.

REFERENCE.

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Chapter XII.

INFECTIOUS LARYNGOTRACHEITIS OF FOWLS.

The virus of laryngotracheitis of fowls has been shown to provoke substantial immunity to re-inoculation (Beaudette and Hudson, 1930), and this immunity is associated with the presence of antibody in the blood (Beach, 1931). The virus produces large typical lesions on the chorioallantois and usually gives satisfactory titration counts.

1. Neutralization on the Chorioallantois.

Laryngotracheitis was the first virus to be studied immunologically by the pock-counting method on the chorioallantois (Burnet, 1936). The discussion in this paper tended to suggest that the interaction between virus and serum was of the same type as the phage-antiphage reaction. With further experience with other viruses, we are more disposed to stress the differences than the resemblances between the two types of reaction, and a further series of experiments on laryngotracheitis have been carried out by Burnet and Lush (unpublished). The findings recorded in Burnet's paper have been fully confirmed, but it can now be seen that the results fall closely into line with those recorded for other viruses in the present monograph.

2. The "Time Effect".

As we have found with almost every virus which has been studied sufficiently, the time course of the reaction may take one of two alternative forms. Fig. 26 shows graphically the results of two experiments to exemplify the two types. Each experiment was made by mixing undiluted serum with virus diluted 1 : 10 and testing 0·05 c.c. quantities of the mixture at various times. All the pock counts are plotted against the time after mixing, when they were inoculated into eggs and the averages of the different groups are used for plotting the time curves. There is a fairly wide "scatter" of results in all laryngotracheitis virus experiments, but sufficient numbers of eggs were used in these experiments to give significant results.

In Experiment A., the early counts 0–10 minutes seem to fall into two groups —about 20 and about 35. At twenty minutes and one hour, all the counts are around 17 or 18. This is interpreted as an example where the primary reaction only plays any part, an equilibrium level of about 20 pocks (0·51 p.c.) being rapidly reached. In some of the earlier inoculations, a higher count corresponds to an incomplete primary reaction, as described for influenza.

Experiment C. is a good example of an experiment in which both a primary and a secondary time effect can be seen.

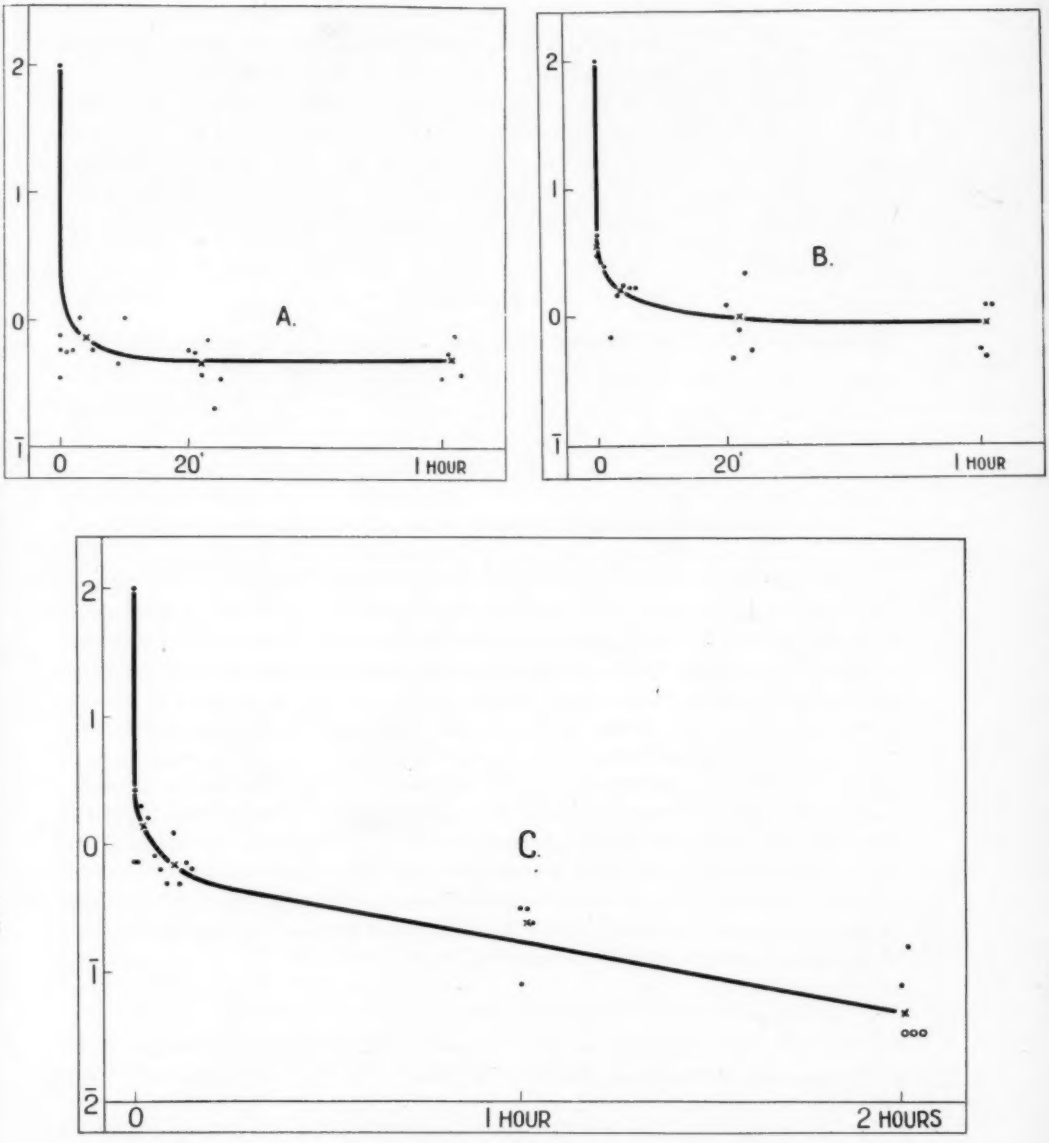

Fig. 26. A., B. and C. Time-survivor curves with laryngotracheitis virus and antiserum. A. and B. show the type of curve in which an equilibrium level is reached. C. shows a progressive inactivation apparently due to secondary processes.

3. Influence of Concentration of Reagents on Results.

In general, the same rules hold for laryngotracheitis virus as for influenza virus, although the results obtained are less conclusive, owing to the larger variation in pock counts and the lower activity of available immune sera. The "C.P = constant" relationship holds approximately over the small range of serum concentrations which can be tested, and the percentage law also holds. Table V of Burnet's paper, reproduced as Table 18, shows these points. Many incidental experiments have given generally similar results. Dissociation by dilution is approximately complete (Burnet, 1936).

Table 18.

Relation between Virus Concentration and the Number of Foci Produced from Immune Serum-Virus Mixtures (from Burnet, 1936).

Serum final concentration.		Time of contact.	Final concentration of stock virus emulsion in mixtures. 1 : 2	1 : 4	1 : 8	1 : 16	1 : 200
Immune	1 : 4	1 –1½ hours	21±, 26, 8	11, 1+, †	5, 4, 2		
„	1 : 10	2¼–2½ „		27, 25, 19±	9, 12, 8+	7, 4±, 5	
Normal	1 : 4	3 „					40+, 62, 64, 59±, 16

Virus: B.A.I. (American) strain. Serum: H·5 from a fowl immunized with strain 8·2.

4. Modification of Pock Character in Partially Neutralized Mixtures.

This virus is the only one which, in our experience, gives, almost without exception, abnormally small foci from partially neutralized mixtures. Vaccinia, which produces large foci, somewhat similar to those of laryngotracheitis, shows no indication whatever of this. Particularly in mixtures that have been in contact for some time, nearly all the foci are small and "weak", i.e. without the character given by the marked ectodermal proliferation and necrosis of the normal pock. Occasionally one finds an egg inoculated with such a mixture in which the pocks, though reduced in number to the usual extent, are completely typical in appearance. It may be relevant to note also that in certain batches of eggs abnormally small foci are produced in the absence of serum. We could find no evidence of latent laryngotracheitis infection in birds from the farm supplying our eggs, and we are inclined to think this effect is a seasonal one, rather than due to small amounts of maternal antibody in the egg.

5. Differences between Virus Strains.

A difference which may be of some general significance has been observed between virus strains obtained (A) from epizootics with severe symptoms, (B) from birds sporadically infected in a region free from epizootics. The epizootic strains (A) were of high virulence for half-grown cockerels, and produced egg membrane lesions with a distinct necrotic centre. The sporadic Victorian strain (B) was practically non-virulent for cockerels, although it immunized effectively: on the

egg membrane it produced pocks of more uniform texture without the central necrotic area or crater. Immunologically the sera obtained from fowls inoculated with either type appeared to be identical, but there were distinct differences in the readiness with which the two strains were inactivated. Irrespective of which type serum was used, the epizootic strains were more readily inactivated than the sporadic strain (Burnet, 1936, Table III, p. 692). After the strains had been maintained by egg passage for more than a year, the same differences were still evident, though perhaps slightly less distinct. The percentage reductions with the serum used were: A. (epizootic), 0·275 p.c., and B., 0·9 p.c., as compared with the previous ratios, 0·16 p.c. to 1·4 p.c., and 0·2 p.c. to 1·0 p.c. shown in the paper referred to.

6. Discussion.

The general principles discussed in earlier sections are directly applicable to the results with the virus of infectious laryngotracheitis. Only two features call for special consideration: (1) the difference in susceptibility to serum shown by the epizootic and sporadic strains, and (2) the modification in the appearance of pocks in the presence of immune serum.

(1) In the discussion of absorption results with anti-influenza sera, we made use of the concept, borrowed from bacteriophage theory, of B. groups on the virus particle surface. These were atomic patterns, antigenically active, but particularly adapted to make intimate contact with constituents of the susceptible cell. In bacteriophages the corresponding entities have been shown to vary in their "avidity", the readiness with which they combine with antibody and bacterial antigen (P.I.A.) varying in parallel (see p. 266, Bacteriophage). The present phenomenon that the strain which is *more virulent*, both for embryo and grown fowl, is more readily inactivated than the less virulent falls satisfactorily into line with these observations and deductions. Increased virulence would naturally be associated with greater avidity both for cell constituents and for antibody.

(2) The small atypical pocks obtained from virus mixtures almost completely inactivated by serum probably represent lesions, the initiation of which has been delayed. One cannot imagine an inheritable change being induced in virus particle partially coated with serum, and once primary multiplication has occurred in the first infected cell, the subsequent development of the lesion should be independent of anything which happened to the original initiating particle. For reasons discussed on p. 307 we cannot conceive that the delay in initiating the lesion is due to the failure of the semi-coated particle to enter a susceptible cell. Such a particle would probably become completely reactivated by dilution within an hour or two, unless it entered a cell. The delay then must be ascribed to difficulties encountered by the virus within the susceptible cell on account of its partial coating of antibody. It is at least suggestive that vaccinia virus, whcih is known (Herzberg, 1936, Haagen and Kodama, 1934) to multiply in the cytoplasm, shows no

such delay; while laryngotracheitis virus, which produces very large intranuclear inclusions, and probably multiplies within the nucleus, shows evidence of considerable delay. It seems reasonable that a virus partly coated with antibody should find increased difficulty in reaching the nucleus of the cell into whose cytoplasm it has been taken. The characteristic increased "stickiness" of antibody-coated particles would probably have this effect, and the delay would not be evident in a virus like vaccinia, capable of multiplying anywhere within the living cytoplasm.

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Chapter XIII.

MYXOMATOSIS OF RABBITS.

Very few recoveries from myxoma infections in rabbits and no effective method of immunization were reported until Shope (1932) found that rabbits which had recovered from infection with his fibroma virus were relatively resistant to myxoma and usually recovered after a more or less severe illness. This has been confirmed by Hyde (1936) and Martin (1936). Shope (1932) reported that sera from such doubly immunized rabbits contained antibody capable of neutralizing the virus *in vitro*. When large numbers of rabbits are exposed to contact infection with myxomatosis, a few spontaneous recoveries occur (Martin). This has also been the experience of Dr. L. B. Bull (personal communication) and most of the experiments to be reported here have been done with serum from one of his experimental rabbits which recovered after showing typical symptoms. Serum A. was taken as soon as the rabbit had regained condition, serum B. three weeks after a large test inoculation of virus subcutaneously. The virus develops readily on the chorioallantois, and pock counting methods can be used for its titration (Lush, 1937). Neutralization experiments on the egg membrane were carried out according to the usual technique, one hour's contact of mixtures at refrigerator temperature being used as a routine.

1. Results of Neutralization Experiments on the Egg Membrane.

Detailed serum concentration-survivor curves have been worked out for sera A. and B., and Fig. 27 shows the combined results of several experiments with each. There is the usual small systematic disagreement between different experiments, probably referable to inaccuracies in the control counts, but the general shape of the curves is clear. Curve A. falls reasonably close to the usual straight line at 45°, but there is an indication of flattening at its commencement. Curve B. shows that the serum of the second bleeding is active to a considerably higher dilution than the first, but that there is relatively little increase in activity as the concentration of serum is increased from 1 : 100 to undiluted. The curve thus resembles those obtained with vaccinia virus, and must be interpreted as indicating the existence of a proportion of virus particles extremely resistant to serum inactivation. Dilution experiments made with Serum A. also indicated a resemblance to the behaviour of vaccinia virus, since dissociation was incomplete. A mixture of virus plus serum 1 : 10 tested undiluted gave counts showing 0·67 p.c. of survivors, diluted 1 : 10, 2·2 p.c., and diluted 1 : 50, 5·4 p.c. of survivors.

No significant "time effect" has been demonstrated in several experiments with different dilutions of Serum A., not even by the method of comparing dilutions made at once after mixing and after an hour's contact.

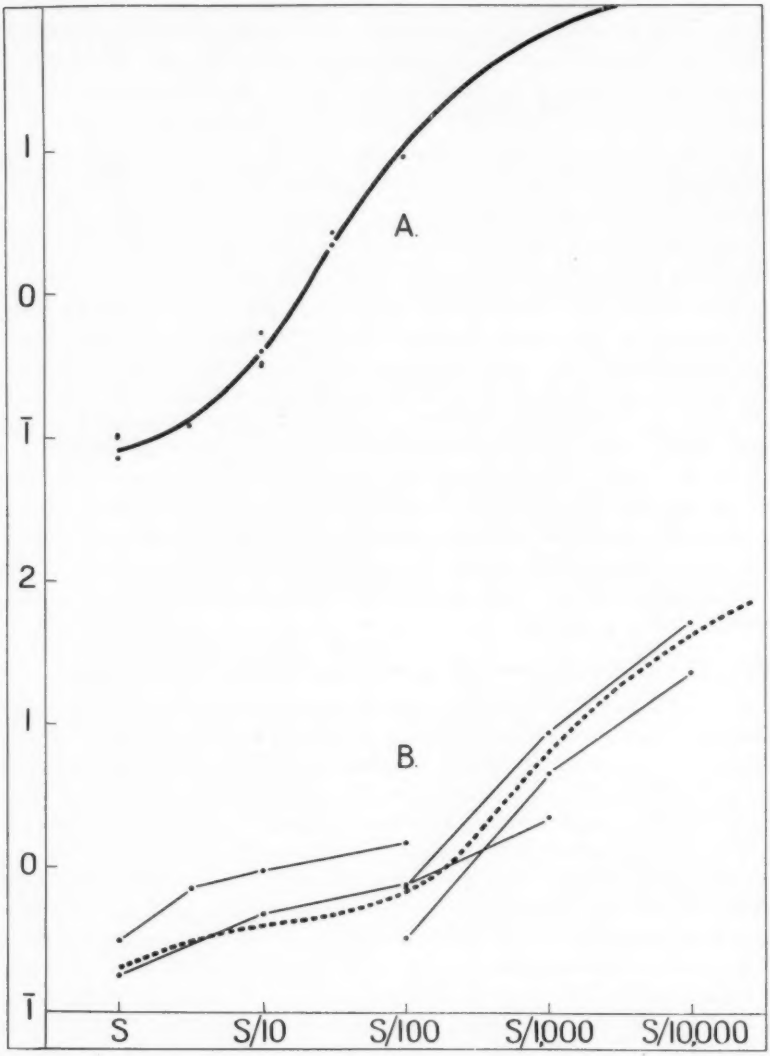

Fig. 27. Serum concentration-survivor curves of myxomatosis virus with serum, A. from a convalescent rabbit, B. from the same rabbit after hyperimmunization. Curve A. from several concordant experiments. Curve B. combined from four experiments, the results of which are shown by fine unbroken lines.

Serum concentration-survivor curves of the same general character as that given by serum B. have been obtained with two other sera from recovered rabbits. One of these was infected spontaneously by contact with another animal. The other became infected during a course of injections of virus, thought to be completely inactivated photodynamically with methylene blue. It recovered after a severe typical illness.

Two sera from rabbits inoculated intratesticularly with Shope fibroma virus, taken after the tumours had retrogressed, showed well marked power to inactivate myxoma virus. Tests on the egg membrane showed reduction to 0·4 p.c. and 0·1 p.c. respectively. No detailed studies, however, have been made with these sera.

2. Discussion.

The salient points of these reactions are (1) the absence of any indication of progress of the reaction with time, even by the dilution method, (2) the incompleteness of dissociation by dilution even when dilution is made very soon after mixing, (3) the flat curve with the stronger concentrations of serum, indicating that a proportion of the virus particles is almost completely resistant to serum inactivation. There is thus a very considerable deviation from our ideal case in the same general direction as we found for vaccinia virus neutralization.

Analysis of the results indicates at once that a relatively high degree of heterogeneity amongst the virus particles must be assumed. The long, almost flat portion of the serum concentration-survivor curve for B. can only mean that with that serum about 1 p.c. of the particles are almost completely resistant to inactivation. In other words, although every antigenic group with which the antibody present is capable of uniting is blocked, a proportion of virus particles is still capable of initiating infection. Further, the fact that dissociation by dilution immediately after mixing is incomplete provides additional evidence of virus heterogeneity. A rapid pseudo-equilibrium is apparently reached in which a considerable proportion of virus particles are permanently inactivated, this proportion not being increased by further contact. Whether or not this rapid irreversible inactivation occurs must, we think, be determined essentially by the differences within the virus particles, otherwise it could hardly fail to be progressive. The remainder of the virus particles, except for the small resistant fraction, are assumed to behave as in our ideal case, developing a reversible equilibrium of antibody molecules, virus particles and antibody-virus compounds.

With further dilution of the serum the immediate irreversible effect becomes much less. This is apparently not due to simple reduction in the reaction velocity by dilution, since even with 1 : 100 serum (A), no clear time effect could be demonstrated. It appears necessary to postulate that the proportion of particles *irreversibly* inactivated on first contact with serum is determined not only by differences amongst the virus particles, but also by the concentration of serum. It is possible that some quite unrecognized factor is at work here. The only tentative

explanation we can offer would be to suggest that for irreversible inactivation two or more antibody molecules must unite simultaneously with an antigenic patch on the virus particle. The likelihood of such an occurrence would be proportional to the square or higher power of the concentration of serum. It is not unreasonable that such a union of two antibody molecules to one antigenic patch should be less readily dissociable and more effectively inactivating, than one to one union by a single "bond". However, in this case, as in the more usual case of the progressive development of irreversibility, hypotheses as to the detailed molecular changes which take place are mere conjectures.

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Chapter XIV.

SUMMARY OF THE IMMUNOLOGICAL REACTIONS OF ANIMAL PATHOGENIC VIRUSES.

1. Neutralization Reactions.

There is no particular novelty in the views we have put forward. A majority of the workers in this field who have expressed any general opinions have held that virus neutralization reactions are manifestations of virus-antibody union. Most have been cautious enough not to attempt any more detailed analysis of the conditions. We feel that the accumulation of data by the egg membrane technique has gone sufficiently far to allow us to attempt such an analysis. In the foregoing sections the evidence has been provided that the viruses of vaccinia, influenza, louping ill, laryngotracheitis of fowls, Newcastle disease of fowls, and rabbit myxomatosis conform in their neutralization reactions more or less closely to the hypothetical ideal case. This was developed on the assumption that virus and antibody combined reversibly, and that the infectivity or otherwise of a given virus particle was determined by the amount of antibody united to it at the time of effective contact with the susceptible cell. Each virus studied in detail has shown some points of individuality, but with all there has been a general conformity to the broad outlines of the ideal case. In addition to our own studies with the chorioallantoic methods, we have reviewed the large amount of recent work on the immunological reactions of vaccinia virus and endeavoured to correlate the results of *in vitro* tests with those obtained by animal titration methods. As far as we have been able to find, there have been only two theoretical studies of a virus neutralization reaction which give results of the same order of accuracy as those we have obtained by the egg membrane technique. These are by Merrill (1936) on equine encephalomyelitis, and by Findlay (1936) on Rift Valley fever. Both used large numbers of mice and give detailed protocols of their results. In both instances the results, when analysed, fall closely into line with the conceptions developed in Chapter V. So also do the results we have obtained in more limited studies on fowl-pox, canary-pox and ectromelia on the egg membrane. None of the antisera to these viruses was sufficiently active to provide fully satisfactory experiments.

On a basis of the data presented in the preceding sections we may summarize our interpretation of the neutralization reaction with animal-pathogenic viruses as follows:

1. Virus inactivation by immune serum results primarily from union of antibody to the virus surface. This union is a reversible one; it takes place at a rate and

reaches an equilibrium determined by the ordinary laws of reversible chemical unions.

2. Union of antibody has no intrinsic inactivating effect on the virus; the inactivation is a result of the interaction between susceptible cell and antibody-coated virus particle. Certain susceptible cells are protected against infection by lesser degrees of antibody coating on the virus particle than are required by other types of cell.
3. In practice, the experimental results obtained with any given virus will deviate from the theoretical values, owing to the operation of one or more of the following factors:
   (a) Wide range of variation in the virus population in regard to susceptibility to inactivation by serum.
   (b) The time required to reach equilibrium may be greater than that experimentally practicable.
   (c) The lag between the time of inoculation and the initiation of an eventually demonstrable lesion by the primary infection of a susceptible cell may vary, both in regard to the tissue inoculated, and from individual to individual.
   (d) Secondary reactions, particularly with concentrated reagents, may occur *in vitro*—aggregation, secondary irreversible or less readily reversible antibody-virus union, and non-specific inactivation by environmental factors.
   (e) When mixtures of virus with relatively large amounts of serum are inoculated, a corresponding degree of local and general passive immunity is produced which may prevent initiated infections developing to demonstrable lesions.

2. IN VITRO AGGREGATION AND COMPLEMENT FIXATION REACTIONS.

We have already indicated that the aggregation and complement fixation reactions of viruses fall into line with the reactions of other antigens, the viruses finding a place in accordance with their particle size between soluble antigens and bacteria. The only aspect which requires final discussion is the question raised by Sabin (1935) and others as to whether neutralizing antibodies are the same as those responsible for the aggregation reactions. In the course of these studies on the immunology of viruses, it has gradually become clear to us that the question is rather an unreal one in the light of modern immunological theory. There is no such thing as *an* antibody: every immune serum contains a population of modified globulin molecules which, considered as a whole, possess a complementary specific reactivity toward all the determinant groups of the antigenic molecule or particle. But each modified globulin molecule carries specific patches corresponding only to a proportion of the total of antigenic determinants. Further, such specific patches,

even those directed toward a single determinant group, appear to vary in quality from molecule to molecule. Serum from an animal after one injection only of antigen may differ considerably from that obtained after a long course of injections of the same antigen. This variation within the antibody population allows us to sort out by appropriate absorptions with related heterologous antigens a wide range of immunological reagents from an antiserum provoked by a single antigen.

A virus particle is much more complex than any single molecular species, and its surface architecture almost certainly varies with its functional state. Multiplying virus, resting virus, heat-killed virus, soluble antigen derived from virus, each will have its own specific architecture superimposed on the antigenic ground plan common to all. Specific patterns on antibody molecules will be correspondingly complex.

When we turn to the other aspect of antibody-antigen relationship, the reaction between formed antibody and the virus *in vitro*, we find the various complexities which have been discussed in other sections. Undoubtedly some antibody molecules in a given serum are more effective than others in inactivating a virus. We have shown that only a proportion of influenza antibody molecules are effective in preventing infection on the egg—a much larger proportion can function to prevent infection in mice. Wilson Smith's (1936) results would suggest that another range of antibody molecules, those common to swine and human virus strains, can function in complement fixation tests. Yet, on the whole, egg, mouse and complement fixation titrations of the antibody in a collection of convalescent sera give concordant results, and it is unnecessary to assume that each reaction corresponds to one particular independent antigen and its corresponding antibody. Ideas are still rather nebulous about the significance of "high grade" and "low grade" antibody, of avid and non-avid antibody, and of highly specific and less highly specific sera, obtained in response to one antigen, but there is no doubt at all that real differences in antibody molecules are responsible for the phenomena which have given rise to these terms.

In regard to virus antisera, our general point of view is that the living functioning virus particle is the most complete antigen, producing antisera of "high grade" or "high avidity" and providing the most complete array of determinant groups. Other virus antigens tend, on the whole, to give less effective antisera, but it is impossible, on present knowledge, to analyse the nature of the diminished efficiency in any detail. The available evidence indicates that aggregation and complement fixation reactions can occur with antibody of lower grade than is effective in neutralization reactions. It does not provide any evidence that high grade antibody is not equally or more effective in producing the *in vitro* reactions.

Future work *may* succeed in delimiting virus antigens as distinct as, for example, H. and O. antigens amongst the Salmonellas, and in showing that one or other is predominantly concerned in neutralization reactions. At the present time,

however, we think that the point of view we have outlined will be found of greater value in interpreting such phenomena as the complexities which are rapidly becoming apparent in regard to the influenza viruses and the results of immunizations with killed virus and virus products.

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Chapter XV.

VIRUS IMMUNITY FROM A BIOLOGICAL POINT OF VIEW.

Immunology at the moment is treated alternately as a branch of physical chemistry and as a highly specialized department of biology. Both points of view are probably as inevitable here as in any other special field of biology, which is susceptible to detailed investigation by physicochemical methods. Further, both aspects must necessarily be considered in more or less isolated fashion until the physicochemical interpretation of biological phenomena comes much closer to the problem of adaptive variation than it has done up to the present. Most of this review has been concerned with the interaction of antiserum and virus which has been considered as far as possible in physicochemical terms. But to obtain any broader understanding of the process, it will be necessary to discuss the process of infection of a vertebrate host by a virus from the most general biological standpoint, the ecological one.

If we consider any common infectious disease of man or animals, measles in man, and psittacosis in parrots, for example, the first point to be kept in mind is that both the host and the virus species persist from generation to generation. Further, over a long period, the extent and character of the disease remain fairly constant. In other words, as far as concerns their interaction, both species of organisms continue to survive and maintain an approximately constant equilibrium. It is the task of epidemiology, with its modern ecological bias, to elucidate how this survival of both species is mediated.

It has been pointed out by a number of authors, e.g. Theobald Smith (1934) and Dudley (1935) that from the point of view of a parasite it is usually a real disadvantage to its species that the host should die as a direct result of the parasitization. There are exceptions: healthy animals may frequently be infected by anthrax spores which have persisted in the carcase of a fatally infected beast; but as a rule, death of the host stops the possibility of further spread of the virus. From the point of view of the continued survival of both organisms, the ideal arrangement is that infection of the host should result in active multiplication of the virus, but no serious damage to the host, and that the host should, for some time, liberate virus into the environment and allow infection to occur in new susceptible hosts. Modern epidemiological work is revealing more and more instances in which such a balanced condition with very little overt indication of disease, is the standard type of infection, the "typical illness" being actually a rare accident. If we are to look for an adaptive significance, e.g. in certain immunological pheno-

mena, we must first be certain that the assumed adaptation is relevant to the usual processes by which the virus survives. As a hypothetical case, from modifications of which the actual conditions in specific infections may be derived, the following account may serve. Virus from the environment comes into contact with susceptible cells of the host, multiplies therein, and is liberated in increased amount from the damaged cells. Further cells are infected, but soon this process is checked, either immunologically or by some other method, so that only a small proportion of cells, well within the reparative capacity of the host, are involved at any one time. Sufficient infection persists, however, to maintain the host as a source of virus from which other animals may be infected for a long enough period to ensure that on the average each infected host passes on the infection to one other susceptible individual.

Which of the known properties of the two organisms are adapted to perpetuate such a cycle as this, and from what variations in these do the observed deviations of the cycle from the ideal, derive? An attempt to answer these questions seems to us the most fruitful way to reach an understanding of the biological significance of the various immunological phenomena which have been demonstrated in regard to virus diseases. With certain at least of the virus diseases it has been obvious for centuries that after one attack the individual is completely immune to that disease. The whole of the last 40 years' research on the problems of immunity points to an intimate relationship between the fact of immunity to a specific disease and the appearance of antibody in the circulating blood. The relationship may not be a direct one, and there is no lack of puzzling or positively misleading complications; but there is also no reasonable ground for doubting that all acquired immunity, which is specific for a certain micro-organism or toxin, is a result of the development of corresponding antibody.

On the side of the host, then, the development, distribution and persistence of antibody will play a major part in shaping the details of the interaction between the two species. The virus in its turn cannot be regarded wholly as the passive victim of the antibody, it has had to establish a *modus vivendi* in an environment which periodically contains antibody directed specifically against it. In all probability, as will be shown later, the difference between the immunological reactions of viruses and bacteriophages may be related to the adaptations of the former to retain potential infectivity in the presence of antibody, adaptations which are uncalled for in the normal environment of bacteriophages.

Still dealing with our hypothetical ideal infection, we may consider *a priori* what type of interaction between virus and antibody would best serve the interests of *both* species, virus and host. The obvious answer is an interaction which would effectively protect the host's own tissues from being seriously damaged, but not diminish the infectivity of the virus for other individuals. Two such mutual adaptations may be recognized in the characteristics of virus immune reactions as

they are observed in certain experimental infections. In the first place, the high degree of reversibility by dilution of virus-antibody reactions can be regarded from this point of view. Virus liberated from a partially immune host will in general be associated with, and perhaps united to, antibody. If, when the infecting material is diluted in the tissues of a new susceptible host, the virus is automatically freed from antibody, then such a characteristic is of distinct survival value for the virus species. In the immunized host, the circulating antibody will maintain all virus particles not actually within cells in a partially coated and relatively innocuous condition. When such antibody-coated particles, however, reach the blood, saliva or nasal secretion of a susceptible non-immune host, there will be, if our experiments are any guide, dissociation of the virus antibody union by simple dilution, and the virus will regain its infectivity.

The second possible adaptation to this end has been demonstrated only with vaccinia virus, but may be a factor in infections of other types as well. This is the ability of the virus to liberate relatively large amounts of soluble antigen in the actively infected tissues. It is probable that this liberated antigen has the same "protective screen" function that has been demonstrated for the free polysaccharide antigen of the pneumococcus, an ability to "mop up" antibody before it can reach the virus particles themselves. The soluble antigen will clearly be in greatest amount in the necrotic cells most likely to be cast off into the environment and to serve as infecting material for a fresh host. It may be regarded as mainly an adaptation to facilitate virus proliferation within the host, but will incidentally be of assistance in helping transfer of infection to a new host.

It may be objected that we are claiming as adaptations what are merely simple physicochemical consequences of the interaction between particles of the size and chemical nature of viruses and antibody. An adequate answer to this objection may be found by contrasting the immunological behaviour of bacteriophages with animal viruses. Both sets of entities are of the same order of size, and have obvious resemblances of chemical composition and biological behaviour; but as we have shown, there are very distinct differences in the nature of their reactions with homologous antisera. The union of bacteriophage and antibody is steadily progressive, and almost completely irreversible by dilution. On the other hand, the four viruses, vaccinia, influenza, louping ill and laryngotracheitis, all show a common high degree of reversibility. These four are distinct enough in regard to particle size, mode of spread and type of lesion produced to suggest that any character they have in common is likely to be possessed by the great majority of other viruses as well.

From our present ecological viewpoint, the difference in this respect between bacteriophages and animal viruses is only to be expected. The normal existence of a bacteriophage species is in no way influenced by antibody. The intravenous injection of the bacteriophage in a rabbit is a quite unnatural happening, and the

immunological response to it is the automatic response of a mechanism adapted to deal with other more important types of micro-organism. The type of interaction between phage and antibody then may be regarded as biologically neutral; there is no element of adaptive compromise between the two agents.

Adaptive variation must have a physicochemical basis, and it is interesting to speculate on the nature of the differences between viruses and bacteriophages responsible for the contrasting types of union with antibody. The simplest interpretation is that antibody-virus union, which resembles antibody-simple haptene union in its high degree of reversibility, is, like this, primarily a matter of union of one antibody molecule to one determinant group only. With bacteriophages, antibody would, on the contrary, be capable of union by more than one linkage. For instance, if one of the main antigenic components on the phage surface was chemically similar to the polysaccharide of Pneumococcus III with numerous aldobionic acid units in the molecule, each capable of acting as a determinant group, such a condition could be easily realized.

As we have previously suggested, the possibility of a single antibody uniting to a virus particle at more than one determinant group will be small, unless the determinant groups on the virus particle surface are numerous and of only a few specific types.

It may be suggested that outside the living susceptible cell the virus particle has a relatively inactive surface with only small numbers of antigenic determinants presenting. This does not mean that virus particles remain in this condition when they are actively multiplying within the cell. Dale (1935) has suggested that the free virus particles, which are the only ones we can investigate immunologically, may be different in many respects from the particles of the same virus in the course of active intracellular multiplication. Like bacterial spores, they may represent stable chemical systems requiring no expenditure of energy to maintain their organization. But within the cell they are obviously intensely active metabolizing systems, multiplying at least as rapidly as bacteria. It is then perfectly reasonable to consider that the liberated particle presents a non-reactive surface to the environment with few of the "side chains" capable of acting as antigenic determinants. Yet within the cell there must be a great change in the surface reactivity of the particle to allow the necessary interactions with cell metabolites which are needed to provide material for virus multiplication. Such an activated surface could hardly fail to be much more *antigenically* active also than the quiescent surface. Such an hypothesis would help to explain why infection by a virus provokes a far more substantial immunity than non-infecting vaccination procedures, and why the *in vitro* union of a virus with antibody is so readily reversible, despite its obviously high antigenic power in the course of infection. Influenza virus exemplifies these points well. Following infection, a ferret becomes completely immune

and develops a high titre of circulating antibody, but inoculation of living virus by other routes than the respiratory one does not result in infection or immunity. With large doses, circulating antibody appears and a slight degree of active immunity (absence of lung lesions) is evident, but no ferrets are completely protected (Smith, Andrewes and Laidlaw, 1935). Finally, as we have shown, the *in vitro* reaction with antibody is completely dissociable by dilution.

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Chapter XVI.

THE SITE OF FORMATION OF VIRUS ANTIBODIES.

The point of view developed in the last section that virus proliferating in a susceptible cell was in an activated state and probably more highly antigenic than when in the resting state, brings into prominence the question as to where and how virus antibodies are elaborated. Our ignorance on this point is in part due to the absence of any adequate knowledge of the nature of antibody production in general, but there are some less fundamental difficulties in regard to virus immunology which are perhaps susceptible to experimental study. It is currently accepted that antibody production is a function of mesodermal cells, particularly those of the reticulo-endothelial system. If this holds also for viruses, we encounter difficulties with the fact that certain viruses which have never been shown to infect mesodermal cells, are yet good producers of antibody. There are several possible origins of such antibody to be considered: (1) It may be that any infected cells which overcome the destructive action of the virus themselves produce antibody. (2) Virus may pass from the breaking down susceptible cells to the adjacent inflammatory cells, and antibody production may take place either in the immediate neighbourhood of the infected tissue, or (3) in the regional lymph glands, as is suggested by McMaster and Hudack's (1935) experiments, with paratyphoid bacilli and vaccinia. Finally (4), virus may pass in the blood stream to the accumulations of reticulo-endothelial cells in the spleen, liver and bone marrow, and there provoke antibody formation. With viruses which are capable of infecting a wide range of tissues, e.g. vaccinia, yellow fever and psittacosis viruses, the virus circulates freely in the blood at some stage of the infection, and the whole reticulo-endothelial system may well be involved in antibody production. Local formation of antibody is likely to be demonstrable only with those viruses which can infect only one particular tissue of the susceptible host, and we shall briefly discuss the conditions under which immunity develops against one or two such viruses.

Recent work has shown that many viruses, originally thought to be strictly confined to one tissue, e.g. the nervous system, are capable of multiplying in cells of other type, but there are still certain viruses which are generally considered to infect only one class of tissue. Rabies and poliomyelitis have never been shown capable of infecting cells other than those of the nervous system, and with both diseases spread within the body is by nerve pathways only. Clinicians have cast some doubt on the rigid neurotropism of poliomyelitis virus in the human subject, but the recent demonstration by Sabin and Olitsky (1936) that the virus would grow in human embryo brain cultures, but not in cultures of any non-nervous tissue, provides strong support for the orthodox view.

The virus of epidemic influenza can infect only the respiratory passages of the susceptible laboratory animals and probably of man also. Inoculation of ferrets by any route not involving the respiratory tract does not result in infection, and produces little or no increase in resistance to infection, although a certain production of antibody may result. There are no reports available as to whether the virus circulates in the blood during infection. The virus of infectious laryngotracheitis in fowls is also limited in its action to the laryngeal and tracheal epithelium in normal circumstances, but the epithelium of the cloaca or some of its appendages can be experimentally infected. Both influenza and laryngotracheitis viruses can infect the ectodermal cells of the chorioallantoic membrane, and adapted influenza virus can almost certainly also infect vascular endothelium (Burnet, 1936b), so that in embryonic tissues at least there is a wider range of susceptible cells.

There are certain aspects of active immunity to viruses of such limited tissue specificity which bear on our problem of the site of formation of virus antibody. Poliomyelitis may be considered first. When a monkey recovers from a paralytic attack of the experimental disease it is completely immune to intracerebral or intranasal re-inoculation with the same strain of virus, and shows virus inactivating antibody in its serum. On the other hand, when animals are given a prolonged series of intradermal injections of virus they develop circulating antibody in considerable amount but are still normally susceptible to infection by virus administered intranasally.

From these experiments we have clear evidence that the presence of circulating antibody is in itself insufficient to protect the animal against intranasal infection. There is very good ground for assuming that all "specific" immunity is dependent on the existence of antibody, either circulating or in the tissue fluids, plus the power to produce further supplies of antibody with increased rapidity on restimulation by the specific antigen. To account for the differences between the two cases we are almost compelled to postulate a local accumulation of antibody, or of the antibody-producing mechanism at some "strategic points" in relation to the central nervous system. The details of these local mechanisms must be left for future research to elucidate.

Amongst human beings epidemiological evidence indicates conclusively that most children in urban communities become infected with the virus and develop an immunity shown both by the appearance of circulating antibody and by insusceptibility to infection in adult life.

The detailed pathology of the subclinical infections responsible for such immunization is, however, completely unknown. Current views incline toward the view that an infection of the specific olfactory cells, which are the only nervous cells in immediate contact with the environment, occurs. The virus multiplies sufficiently to produce an immunity response, but does not spread further into the central nervous system. Burnet (1936a) produced some evidence in favour of

such a conception from analogy with the phenomena observed in rats infected intranasally with louping ill, and Olitsky, Sabin, and Cox (1936) described a somewhat similar phenomenon in response to intranasal inoculation of vesicular stomatitis virus in mice. Whatever the nature of the immunological response in the child, it is efficient enough to ensure that all subsequent contacts with the virus produce no more than subclinical infection. That this degree of protection is important can be deduced from the high adult morbidity and mortality when poliomyelitis attacks a completely "unsalted" community, as occurred in certain Pacific island epidemics. There is a strong suggestion from all the clinical and experimental evidence that such immunity may be largely concentrated at the point of natural attack—the olfactory mucosa. Is the antibody produced, if not in the infected olfactory cells themselves, then in the adjacent submucosal cells, the local region retaining both formed antibody and the power to produce more antibody if need be? There is no direct evidence to justify an affirmative answer, nor, on the other hand, is there anything biologically improbable about the suggestion. The discovery of some neurotropic virus infection of laboratory animals, which shows the same high infectivity and low morbidity as human poliomyelitis, would allow an experimental test of the hypothesis. Unfortunately there seems to be no technical possibility of studying the question with poliomyelitis virus itself.

Turning to the two viruses infecting particularly the respiratory tract, we find again that neither infection nor immunity can as a rule be induced if living virus is administered by any other route. Natural immunity following infection is absolute for some months at least. It may be significant that when subclinical infection by an attenuated strain is induced the resulting immunity is just as solid as after infection by a virulent strain. This has been shown in this laboratory for both laryngotracheitis (immunization with the almost avirulent Victorian strain —Burnet, 1936b) and in greater detail for influenza virus. Egg-adapted influenza virus is almost completely avirulent for mice and ferrets, but immunizes them solidly against infection by a virulent strain and provokes high antibody titres. The infection induced in ferrets by egg virus is not contagious, and general considerations suggest that little virus would be liberated, either into the respiratory passages or into the blood stream. Again, it appears likely that the bulk of antibody production takes place in the infected organs, possibly in the cells directly invaded by the virus, more probably in the accumulations of lymphocytes and other mesodermal cells in the immediate neighbourhood of the epithelium. Should this be the case, it may have an important bearing on the method of artificial immunization to be adopted with human beings. Intranasal instillation of attenuated virus would, on the hypothesis of local antibody production, provide a more effective immunity than any method employing subcutaneous or intramuscular inoculation. The conditions are complicated, however, by the fact that practically all human beings possess a basic immunity acquired by natural clinical or subclinical infection with influenza. Although a normal ferret cannot be

immunized against influenza by parenteral administration of virus, a recovered animal whose immunity has fallen below an effective level can be rendered re-immune by intramuscular administration of virus. In such a case we can imagine that circulating virus or virus-components may provoke an active secondary production of antibody in the initially affected tissues of the respiratory passages. *A priori* considerations can therefore carry little weight in deciding what is the method most likely to provide a practicable means of preventing epidemic influenza.

It is to be hoped that an experimental study of this question of the site of origin of virus antibodies will be made with influenza virus. With the pig, ferret, mouse and developing egg available as experimental animals, there are no basic technical difficulties such as prevent such studies with poliomyelitis.

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Chapter XVII.

THE PRINCIPLES OF ACTIVE IMMUNIZATION AGAINST VIRUS INFECTIONS.

This monograph has been almost entirely concerned with the phenomena resulting from the interaction of viruses and immune sera and with the theoretical bases of these phenomena. We believe that such problems are of interest to most workers in the fields of immunology and virus research, but in themselves are not immediately relevant to the economically and medically important phases of such work. In general, the practical aim is either to eliminate the possibility of infection from the community, or, if this is impracticable, to develop methods of immunization, in themselves harmless, which will reduce the incidence and severity of disease due to the virus in question. A limited discussion of the aims and methods of active immunization against virus infections in the light of the theoretical conceptions which we have developed may be of some service in providing a theoretical orientation of the problems involved.

It seems axiomatic that no method of artificial immunization will be capable of producing a more substantial immunity than follows a natural infection by the virus concerned. Naturally acquired immunity may vary from the probable lifelong immunity after an attack of yellow fever (Sawyer, 1931) or measles to the year or two of immunity after influenza, or the almost complete absence of immunity to Dochez's virus of the common cold (Dochez, 1933). In each case, all we can hope to do is approach the efficiency characteristic of natural post-infection immunity. This is not to deny the possibility in such a disease as influenza that by suitably spacing re-inoculations with the immunizing agent we may provide a more effective and consistent immunity than could be obtained if the irregularly recurring epidemics of the disease were the only infecting and immunizing possibility. Our point is only that any one short series of immunizing inoculations will in general produce an immunity which may approach, but will never exceed, that induced by a natural infection.

Nearly all the effective methods of immunization against virus diseases utilize living virus and depend on the establishment of a subclinical infection as the immediate stimulus to the production of immunity. To reduce the intensity of infection to the subclinical level, one or more of three methods may be employed:

1. Active virus is introduced into the body by such a route that it produces an infection in some tissue where the pathological changes are of only trivial significance. Examples are the immunization of human beings and monkeys against psittacosis by subcutaneous injection (Rivers and Schwentker, 1934), of

fowls against laryngotracheitis by cloacal inoculation, and of cattle against pleuro-pneumonia by the inoculation of virus into the tail. Probably Francis and Magill's method (1936) of immunizing human beings against influenza by subcutaneous inoculation of culture virus falls into this category, but the efficacy of the method has yet to be proved.

2. Active virus is introduced with an amount of immune serum sufficient to prevent *clinical* infection, but insufficient to prevent any infection at all taking place. Strict quantitative control is obviously necessary in such methods, and since a proportion of severe infections is to be expected, they are practically limited to veterinary or experimental work. Successful results have been obtained against rinderpest in cattle and with African horse-sickness and swine fever. In clinical medicine the prophylactic use of measles convalescent serum in contact children is in principle equivalent to the method of virus-immune serum immunization. The serum is given about half-way through the incubation period, and usually converts the infection into a mild or abortive one, which, however, provides the child with a normal subsequent immunity.

3. Attenuated virus is used to produce a mild infection. This is the classical Jennerian method. In addition to vaccination against smallpox we may instance the method of immunization against yellow fever developed by the Rockefeller Foundation Laboratories (Sawyer, Kitchen and Lloyd, 1931), in which mouse brain virus is used along with human immune serum, so combining methods 2 and 3. The immunization of ferrets and mice against influenza by means of egg-adapted virus (Burnet, 1937) falls in this category, and may eventually be applicable to human beings. As in each of these instances the general principle involved is that if a virus is transferred to and repeatedly passed through a different initially less-susceptible host, its virulence for the original host species is diminished.

With any of these methods the conditions in the body during immunization are essentially similar to those associated with natural infection, the main difference with the second and third methods being a quantitative reduction in the intensity of the processes occurring. When immune serum is inoculated along with virus a certain degree of antibody coating of the virus particles will in the first place diminish the number of cells initially infected and increase the average time elapsing before virus multiplication commences in those cells which are initially infected. The same partial inactivation and delay will be evident in regard to the virus particles liberated from infected and necrosing cells. With the appearance of actively produced antibody the passive effect will be greatly reinforced, and the infection overcome.

As various writers (Topley and Wilson, 1936) have pointed out, the behaviour of an avirulent or weakly virulent micro-organism in a normal animal is often remarkably similar to that of a virulent strain in a partially immune animal. An

attenuated strain of virus is in all probability one which does not so readily enter susceptible cells, and which, once within, does not multiply so rapidly as the virulent strain. There should therefore be no essential difference in the pathology of the mild infections induced by these two methods. The efficacy of the immunity induced will depend (1) on the amount of virus multiplication which occurs before the infection is overcome. This determines the amount of virus antigen available for the stimulation of antibody production. It will be smaller if the amount of immune serum in method 2 is increased, and if the attenuation of virus (method 3) has been carried so far that it is almost avirulent for the inoculated animal. (2) On the similarity in antigenic structure between the inoculated virus and that of the natural infection. With active virus-serum mixture inoculations the two antigenic structures are necessarily identical, but it is by no means certain that attenuated virus retains the precise antigenic pattern of the parent active virus. Probably in almost every instance when a virus is transferred to a new relatively insusceptible host slight antigenic differences appear with development of the new type of virulence. We may instance the case of poliomyelitis, in which Paul and Trask (1933) showed that adaptation of human virus to monkeys was associated with a detectable change in antigenic character. In the conversion of smallpox virus to vaccinia by rabbit passage, Amies (1932) found well-marked changes in the specific agglutinability of the virus particles by immune sera. The change in immunological reactivity of influenza virus following egg passage has been described in Chapter VII. From the practical standpoint these minor antigenic changes in attenuated viruses are probably of little importance. The antibody produced in response to the attenuated strain will possess many active areas corresponding to the antigenic determinants of the active strain and will be effective in minimizing the pathogenic activity of the latter, particularly when it is present in only the small dosage of natural infection.

Vaccination by killed virus is currently held to be the ideal method for the prophylaxis of human infections, since it eliminates any risk either of infecting the inoculated subject or of rendering him a source of contagion for others. Unfortunately, it is found in practice that killed virus preparations are usually very ineffective as antigens. There are obvious reasons for this. The preparations used up to the present have necessarily been composed of extracts or emulsions of infected tissues usually of some other species than that to be immunized. The amount of virus antigen available is only that actually injected, and though the titre of the material was initially high by conventional standards, yet the actual weight of virus antigen present will always be minute in comparison, e.g. with the amount of tissue protein also present. Unless the killed virus is an extraordinarily potent antigen, little antibody production can be expected.

The presence of foreign protein and lipoid in the antigen preparations is a serious practical disadvantage on account of (1) possible sensitization effects on repeated inoculation, (2) reduction in the response to the specific virus antigen,

owing to the competing antigenic stimulus of foreign tissue components. A good example of the importance of this factor is found in the work of Laidlaw and Dunkin (1928) on dog distemper. They found that an effective vaccine for ferrets could be prepared by the action of formalin on infective ferret spleen, but that this vaccine was useless for dogs, while similar vaccine prepared from dog spleen protected dogs but not ferrets. In each case where the killed virus antigen was "swamped" by foreign tissue antigen, no effective response occurred. (3) Occasional paralytic effects, apparently due to lipoid materials in vaccines prepared from nervous tissue. Accidents of this sort have occurred in a small percentage of patients undergoing antirabic treatment. An apparently similar condition can be induced in monkeys by repeated inoculations of normal rabbit brain substance (Rivers, Sprunt and Berry, 1933).

Modern methods of concentrating vaccinia virus (Craigie, 1932; Parker and Rivers, 1935) will provide amounts of killed virus practically free from tissue components, sufficient to overcome these disadvantages, and similar methods may eventually be applicable to other viruses.

Should such purified virus materials become available as vaccinating agents, adequate dosage will doubtless result in the production of considerable amounts of antibody. It remains to be seen how closely this antibody will resemble that produced in response to natural infection. As far as can be determined from the work with killed suspensions of the elementary bodies of vaccinia, the antibodies resulting provide good agglutinating and precipitating agents, but are noticeably inferior to post-infection antibodies in "neutralizing" ability. Considerably more quantitative work on such differences will be required before it is possible to discuss the problem in detail.

Slight differences between the antibodies produced in response to infection on the one hand, and by immunization with killed virus on the other, may be of great interest to the academic immunologist, but in practice they will probably serve the same purpose, provided a sufficient concentration of antibody is developed. It is not necessary that all the antigenic determinant groups of a virus particle be blocked by corresponding antibody for it to be rendered non-infective. In fact, it seems that rather distantly related antibodies may on occasion serve to prevent infection by a natural route. We may recall that ferrets immunized to swine influenza virus are, as a rule, actively immune to infection with human type virus, although *in vitro* neutralization tests with serum indicate that only minor antigenic components are common to both types. If killed virus can be administered in amount sufficient to provoke an antibody concentration equivalent to that following natural infection, there is every likelihood that immunity will be equally substantial. A reservation should be introduced that in the case of infections limited to certain specific tissues, general antibody production may be ineffective for the reasons discussed in Chapter 15.

In practice, killed virus is mainly used to produce a minor grade of immunity which will allow the safe administration of living virus to complete the immunization process. The modern technique of vaccination against dog distemper is a good example of this (Laidlaw and Dunkin, 1928).

There are rather numerous examples in the literature where investigators have found that virus treated with some antiseptic under strictly limited conditions is non-infective, and can produce substantial immunity, but that if exposure to the antiseptic is prolonged no immunization results. Such methods usually fail to give reproducible results, and the immunization is generally regarded as being due to low grade infection by virus remaining viable but rendered less virulent by the antiseptic. Such empirical procedures as the use of cattle plague bile for immunization probably depend on similar principles.

The success of methods of this sort raises some theoretical difficulties. It is hard to imagine that the action of antiseptic on a "resting" virus particle which remains viable should result in a heritable reduction of virulence. Once multiplication within a susceptible cell has commenced, the descendant virus particles should be of normal virulence and, *a priori*, capable of initiating a typical infection. The most likely explanation is that the damaged but still viable particles are (1) present only in small numbers, and (2) when they enter susceptible cells there is an abnormally long lag phase before multiplication begins. The nett result is equivalent to a series of extremely minute subinfective doses of active virus given over a considerable period.

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